Discovery of the new antimicrobial compound 7-*O*-malonyl macrolactin A

Doctoral Thesis

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A Juliana

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Abbreviations

AMP	Ampicillin
ATCC	American Type Culture Collection
°C	Degrees Celsius
CF	Cystic fibrosis
CFU	Colony Forming Units
α	Specific rotatory power
δ	NMR chemical shift (ppm)
λ	wavelength (nm)
µl	10^{-6} litre
µm	10^{-6} metre
CH_2Cl_2	Dichloromethane (see DCM)
COSY	Correlated SpectroscopY
DAD	Diode Array Detector
DCM	Dichlromethane
DSM	German collection of microorganisms
ERY	Erythromycin
EtOAc	Ethyl acetate
EtOH	Ethanol
GEN	Gentamicin
g	gram
IEF	Isoelectrofocusing
HPLC	High Performance Liquid Chromatography

Hz	Hertz
IR	Infrared
L	Litre
m	Multiplet
MeOH	Methanol
MCZ	Miconazole
MIC	Minimal Inhibitory Concentration
MHz	Megahertz
min	Minute
ml	10^{-3} litre
mM	10^{-3} mol
mm	10^{-3} metre
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
NCCLS	National Committee for Clinical Laboratory Standards
NM	Non mucoid
nm	10^{-8} metre
NMR	Nuclear Magnetic Resonance
7-O-m	7-*O*-malonyl macrolactin A
PAE	Post-antibiotic effect
PBS	Phosphate-buffered saline
ppm	Parts per million
RP	Reversed phase
SCV	Small Colony Variants

TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
UV	Ultra Violet
VAN	Vancomycin
VRAR	Vancomycin Resistant Ampicillin Resistant Enterococci
VRAS	Vancomycin Resistant Ampicillin Sensitive
VRE	Vancomycin Resistant Enterococci
WT	Wild Type

1. INTRODUCTION

1.1. Secondary metabolites

Formerly, secondary metabolites were defined as substances with a low molecular weight, which were not products of the primary metabolic pathway of the producing organism. As a matter of fact, it was thought that these products did not have a roll in the microbial primary functions or growth [1, 2]. Therefore, it was thought that production of secondary metabolites did not represent any advantage for the producing microorganism. Contrary, nowadays it is considered that cell investment in secondary metabolite production is almost the confirmation of a function that should give the organisms certain advantage against other members of the microbial community [3]. In fact, secondary metabolites are accepted to be essential for the producing cell as inhibitors of other organisms that compete for the same food supply or as regulators of cellular differentiation processes. In addition, it is reported that they are indeed products of biosynthetic pathways, which have evolved to give these types of advantages [4, 5].

The nature has provided a broad spectrum of structurally diverse secondary metabolites [6-8]. Despite this great diversity, microbial secondary metabolites are synthesised from only a few precursors, in pathways with a relative small number of reactions, which branch from just a limited number of reactions of the primary metabolism [5]. The mentioned structure diversity is reflected in a variety of biological activities as, for instance, inhibitors of enzymes and antitumor, immunosuppressive and antiparasitic agents [4, 9]. Aside their medical relevance, these compounds are used in many industrial, agricultural and forest applications [4]. About 100.000 secondary metabolites of a molecular weight below than 2500 have been characterised, among them approximately 50.000 from microbial sources

[10]. New microbial bioactive products continue to be discovered at an amazing pace: 200-300 per year in the late 70s, increasing to 500 per year by 1997 [4].

Antibiotics are perhaps the most widely studied type of secondary metabolites with 12000 antibiotics known up to 1995. Again, microbial cells are the most importance source of this type of secondary metabolites. Indeed, from the known antibiotics 55 % are produced by filamentous bacteria of the genus actinomyces, 11 % from other actinomyces, 12 % from non-filamentous bacteria and 22 % from filamentous fungi.

Different alternatives for improving production of secondary metabolites with different activities for biotechnological applications have been extensively investigated. Since, biotechnology industry is based on harnessing the metabolic activities of different organisms to produce a wide variety of diverse compounds, which are used by other industries. Two methods have been applied, optimisation of fermentation process and improvement of strains [11]. The microbial production of secondary metabolites is extremely sensitive to environmental factors or culture conditions [12]. For instance, the in vitro production of most antibiotics depends on the composition of the culture medium in which the producer organism is grown. For this reason, medium optimisation has been the standard procedure for optimising antibiotic production.

Overproduction of secondary metabolites is a complex process and the successful development of improved strains requires knowledge of physiology of the microbial producer and pathway regulation and control of the product. Recently, research efforts have been devoted to elucidate, at the molecular level, the regulatory mechanisms involved in the control of the biosynthesis of antibiotics [13]. Most of the work performed in this field has been focused in antibiotics produced by fungi and actinomycetes. For these, a number of biochemical and genetic controls have been described to optimise the levels at

which the antibiotics are produced in a specific media [14].

1.2. History of secondary metabolites

Secondary metabolites have been used for centuries. Microorganisms, and more specifically their products, were used in food conservation and production of wine, cheeses and bread. For example, as a method of preservation, milk was converted to lactic acid to make yoghurt.

In the same form, secondary metabolites with antimicrobial properties have been used for many years. Indeed, moldy cheese, meat and bread were employed in folk medicine to heal wounds [5, 15]. Yet, it was not until 1870s, that Tyndall, Pasteur and Roberts reported the antagonistic effects of some organisms on others. In fact, the antibiotics era began in 1929 with the penicillin discovery by Fleming. However, antibiotic research and industry only flourished after the commercial production of penicillin in the 1940s [5, 16]. In spite of this, until 1970 only two classes of naturally occurring β-lactam antibiotics, penicillins and cephalosporins, were known. Although, with the advent of new screening and isolation techniques, a variety of β-lactam-containing molecules [17] and other types of antibiotics have been identified. In fact, screening of microorganisms for production of antibiotics has provided the cornerstone of antibiotic research programs for the past thirty years. The great majority of such studies have been carried out with fungi and actinomycetes, which are capable of producing natural products with widely divergent chemical structures.

Some peptides have shown antibiotic properties; over 400 with antimicrobial activity have been identified from extremely diverse sources including plants, insects, bacteria, and vertebrates [18]. Some of these peptides are induced at epithelial surfaces in response to

invading organisms. Many of these antimicrobial compounds kill microorganisms by causing membrane permeabilization, although not necessary as their sole mode of action [19, 20]. Finally, several antimicrobial peptides are additionally involved in chemotaxis promoting wound healing. In addition, some contribute to adaptative immunity by mobilizing memory T cells and immature dendritic cells.

Parallel to the screening for new antibiotics, efforts have been focused in finding low molecular weight secondary metabolites with other biological activities. Among others, secondary metabolites with activity as enzyme inhibitors, plant growth stimulators, herbicides, insecticides, antihelmintics and immunosuppressant have been obtained. Two main strategies have been used during the screening process of these compounds. First, the screening of known secondary metabolites which had failed as useful antibiotics and second, screening of unknown compounds using new technologies for detecting inhibition of enzymes and biological activities in other targets.

Secondary metabolites play a significant role in clinical practice due to their activity as antimicrobials used in the treatment of microbial induced infections. But apart from these activities secondary metabolites possess other pharmacological activities useful in the medical field, some act as carcinostatics. The best antitumor substances are mithramycin, bleomycin, daunomycin and adriamycin [16]. Other have properties as anabolics, anesthetics, anticoagulants, anti-inflammatories, immunosupressants (cyclosporin A and tacrolimus), hemolytics, hypocholesterolemics (statin), and vasodilatories.

1.3. Function and importance of secondary metabolites

Several hypotheses exist about the origin and function of secondary metabolites. The most accepted considers secondary metabolites as waste products that under the pressure of

natural selection have evolved as messenger molecules which must endure long enough to shuttle between the various components of the microbial community. This fact, would explain the secondary metabolites tendency to be small organic molecules, as a natural consequence of their functions [21].

The explicit role of secondary metabolites in microbe-microbe interactions is unknown, specially how their production affects the community ecology. In fact, the notion that microbial antibiotics are employed by the producing organisms to adversely affect their competitors is cited. Nevertheless, it is accepted that they are beneficial to the producer not only against microbial competitors but also against other organisms including parasites, plants, insects and even superior animals [5, 22].

An interesting property common to most potent antibiotics is that at very low concentrations, they act as growth stimulants [4]. This raises an interesting possibility that some secondary metabolites may actually stimulate growth of other microorganisms antagonistic to the growth of the competitors of the secondary metabolite-producing organisms. The presence of complex biosynthetic pathways for production of complex antibiotics suggest that they must have an important role in microbial survival, either as inhibitors of other competing organisms or as regulatory effectors during some stage of the cell differentiation process, since sensitive organisms need to evolve only a single enzyme of any several reaction types to inactivate most antibiotics [4].

Nowadays, several arguments support the hypothesis that secondary metabolites improve the survival of the producer in competition with other living species. These arguments are as follows [5]:

a) Secondary metabolites act as an alternative defence mechanism, because only the organisms lacking an immune system are prolific producers of these compounds.

b) They have sophisticated structures, mechanisms of action, and complex and energetically expensive pathways.

c) Secondary metabolites act in the competition between microorganisms, plant and animals.

d) They are produced by biosynthetic genes clusters, which would only be selected if the product conferred a selective advantage. Some particularities of these genes clusters are the absence of non-functional genes and the presence of resistance and regulatory genes.

e) The production of secondary metabolites with antibiotic activities is temporarily related with sporulation when the cells are particularly sensitivity to competitors and requiring special protection when a nutrient runs out.

Furthermore, the wide diversity of secondary metabolites suggests a broad range of functions. Nevertheless, these functions could depend on the conditions, optimal or not, surrounding the producer microorganism. Finally, due to their crucial importance the study and exploitation of secondary metabolites continue to progress despite the lack of agreement regarding why microbes produce such chemical diversity of antimicrobial compounds.

1.4. Genetic of secondary metabolites

The genes regulating and ensuring synthesis of secondary metabolites and their expression can be grouped in 5 classes: I) structural genes, that code for enzymes involved in the biosynthesis; II) regulatory genes, that determine the induction or repression of the structural genes; III) genes that determine the resistance of the producing organism; IV) genes controlling the permeability to the compound and V) genes that control primary pathways. The genetic regulation of all above mentioned genes is highly complicated

because many environmental and microbial factors affect the production of these compounds.

The genes coding for biosynthesis of secondary metabolites have some features that can be summarized as follows:

a) They are arranged in clusters on the chromosome.

b) These genes are organized in several transcription units not in a single operon.

c) Within each cluster at least some transcription units are controlled by the products of pathway specific regulatory genes.

d) Specific regulatory genes are situated in the closest proximity of biosynthetic genes.

e) Gene expression is primarily controlled at the level of transcription but also at the level of translation.

f) Genes coding for resistance to a given metabolite are closely linked with the cluster of biosynthetic genes. Usually genes coding for at least two different types of resistance to a produced secondary metabolite can be found within the gene cluster.

g) Expression of genes coding for a secondary metabolite, including genes coding for resistance to it, is controlled by central overruling regulatory circuits often exhibiting pleiotropic regulatory effects [12, 23].

Functionally related genes are on the chromosome suggesting that at least part of their evolution has occurred as a unit. Evolutionally, two types of gene clusters that produce secondary metabolites can be described: first, a gene cluster might contain many genes, some of which give rise to chemical transformations of substrates and many other that do not. This type of cluster would be indicative of a natural product which has not been selected for and which is not functional. Second, a gene cluster might contain only genes

that code for enzymes of a particular biosynthetic pathway, resistance genes and regulatory factors, and no "junk" genes. This type of gene cluster indicate a natural product that has been selected for and which has had a beneficial function for the producer organism [3, 22].

Since antimicrobial producing genes are tightly clustered in the microbial chromosomes, both gene replacements and coregulation of all the genes necessary for antibiotic formation are facilitated [24]. In fact, by applying DNA recombinant technologies, a large amount of genes coding for enzymes involved in synthesis of secondary metabolites have been cloned in order to improve expression levels. In addition, with the genomic sequences of several antibiotic-producing microbes becoming available, there has been much interest in combinatorial biosynthetic approaches. Indeed, by manipulating the genes of antibiotic biosynthesis pathways, hybrid or variant antibiotics with novel properties and efficacies against resistant pathogens could be generated.

Specifically, the polyketide antibiotics, both the aromatic family represented by tetracyclines, and the macrolide family represented by erythromycins, are produced by clustered genes. In contrast, the aminoglycosides are not produced by corregulated genes, which are dispersed throughout the genomes of the producer microorganism [24]. Finally, contrary to the above-mentioned compounds, peptide based antibiotics as vancomycins, bacitracins, and ß-lactam antibiotics are synthesised on non-ribosomal peptide synthetase (NRPS) assembly lines. However, they also have been considered candidates for combinatorial biosynthesis approaches as their biosynthesis has some similarities with the one of the poliketides [24].

1.5. Microorganisms producing secondary metabolites

As it was mentioned above, secondary metabolites are common to organisms that lack an immune system thus are rarely produced by higher animals [22]. Indeed, these compounds are mostly biosynthesised by bacteria, fungi, algae, corals, sponges, plants and lower animals. Actually, filamentous microorganisms are the main source of secondary metabolites with nearly 75 % of all described antibiotics being produced by actinomycetes and 17 % by molds. Moreover, approximately 40 % of the filamentous fungi and actinomycetes produce antibiotics when freshly isolated from nature. Some isolates of several *Streptomyces* species can produce more than 180 different secondary metabolites [5].

Bacteria that show high natural products production are those belonging to the genus *Bacillus*. Several antibiotics have been isolated from different *Bacillus* strains, moenomycins, difficidins, bacillomycins and bacillaenes are part of this wide variety [25-27]. *Myxobacteria* is yet another genus of bacteria with interesting antibiotic productivities. For instance, Reichenbach *et al*. [28], found that approximately 80 % of the isolated *Myxobacteria* produced compounds with antibiotic activity, many of them with antifungal activity. These data were later confirmed by Foster *et al*. [29] who reported that 77 % of soil Myxobacteria showed antibiotic activity against *Micrococcus luteus*.

An immense variety of active secondary metabolites with different properties have been isolated from several microbial species belonging to a wide range of genera. In Table 1 are listed some of the most important secondary metabolites with biological activities produced by microorganisms.

Table 1. Microbial secondary metabolites with important biological activities

Compound	Producer	Biological activity
Amphotericin B	*Streptomyces nodosus*	Antifungal
Bialaphos	*Streptomyces hygroscopicus*	Herbicide
Cephalosporin	*Cephalosporium chrysogenum*	Antibiotic
Chlortetracycline	*Streptomyces aureofaciens*	Antibiotic, Growth promotant
Cyclosporin A	*Trichoderma polysporum*	Immunosuppressant
Daunorubicin HCl	*Streptomyces sp.*	Antitumoral
Doxorubicin HCl	*Streptomyces peucetius*	Antitumoral
Erythromycin A	*Streptomyces erythreus*	Antibiotic
Gentamicin	*Micromonospora purpurea*	Antibiotic
Kanamycin	*Streptomyces canus*	Antibiotic
Lasalocid sodium	*Streptomyces sp.*	Growth promotant
Lincomycin HCl	*Streptomyces lincolnensis*	Antibiotic
Mitosane	*Streptomyces caespitosus*	Antitumoral
Monensin sodium	*Streptomyces cinnamonensis*	Growth promotant
Oxytetracycline	*Streptomyces rimosus*	Antibiotic, feed additive
Paclitaxel	*Taxomyces andreanae*	Antitumoral
Penicillin	*Penicillium chrysogenum*	Antibiotic
Rifamycin	*Amycolatopsis mediterranei*	Antibiotic
Salinomycin	*Streptomyces albus*	Growth promotant
Spiramycin	*Streptomyces ambofaciens*	Antibiotic

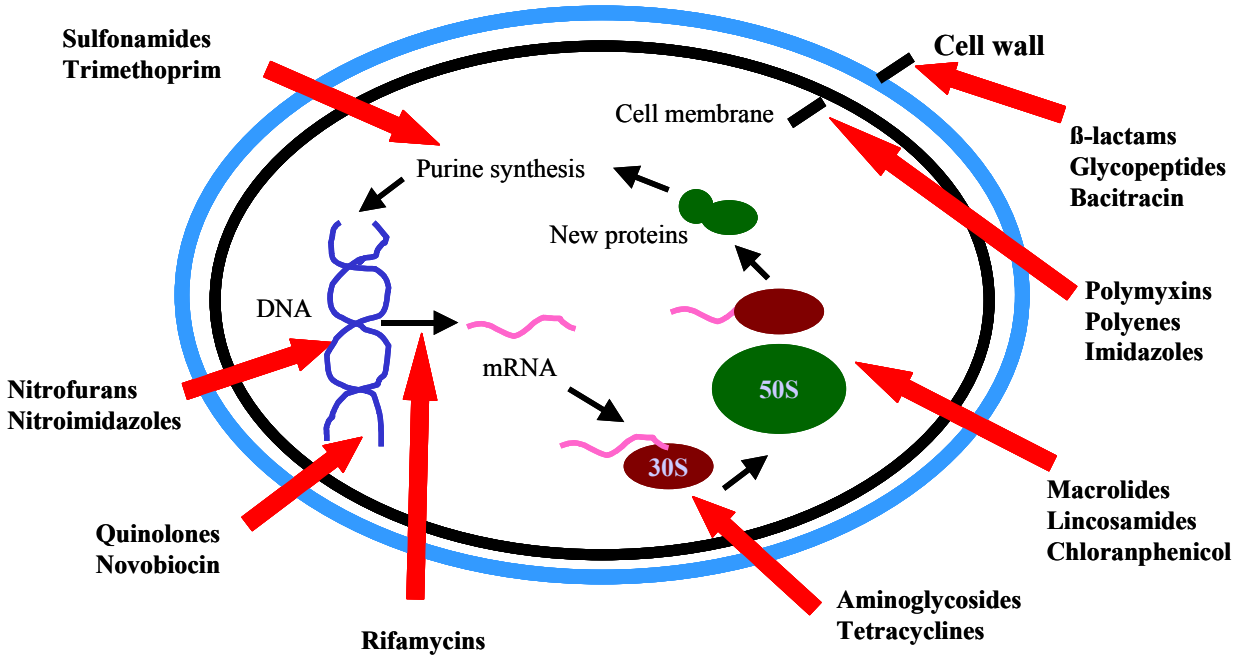
Continued Table 1.

Compound	Producer	Biological activity
Statin	*Aspergillus terreus*	Hypocholesterolemic
Streptomycin sulfate	*Streptomyces griseus*	Antibiotic
Tacrolimus	*Streptomyces*	Immunosuppressant
Teicoplanin	*Actinoplanes teichomyceticus*	Antibiotic
Tetracycline HCl	*Streptomyces aureofaciens*	Antibiotic
Tylosin phosphate	*Streptomyces cirratus*	Growth promotant
Vancomycin HCl	*Amycolatopsis orientalis*	Antibiotic

1.6. Antimicrobials: modes of action

Antimicrobials and specifically antibiotics are define as low molecular weight organic natural products made by microorganisms which are active against other microorganisms at low concentration [4]. This activity develops through a limited number of mechanisms, antimicrobials interfere with cell wall synthesis, cell membrane integrity, protein synthesis, DNA replication and repair, transcription and intermediate metabolism (Figure 1).

Figure 1. Site of action of common antimicrobial agents.



The compounds blocking the cell wall biosynthesis inhibit enzymes involved in synthesis of different components of the cell wall. While the compounds interfering with cell membrane integrity disorganize the structure or inhibit the function of bacterial membranes. Antimicrobials affecting the protein synthesis act impairing the ribosomal subunits, binding to 50S prevents the translation and binding to 30S causes wrong translation, producing toxic and altered proteins. Compounds having an effect on DNA replication and repair, inhibit enzymes as gyrase and topoisomerase and *N*-methyltransferase. Similarly, compounds affecting transcription inhibit the subunits of the bacterial RNA polymerase blocking the entry of the first nucleotide necessary to activate the polymerase. Finally, some antimicrobials interfere with intermediate metabolism by inhibiting enzymes involved in the biosynthesis of different substances. In Table 2 are listed some of the more common classes of antimicrobials and their mechanisms of action

Table 2. Common classes of antimicrobials and their mechanisms of action.

Class	**Family*** / examples	**Mechanism**
β-Lactams	**Penicillins** Methicillin Aminopenicillins	Cell wall synthesis Inhibitors
	Cephalosporins	
	Monobactams Aztreonam	
	Carbapenems Imipenem Meropenem	
Peptides	**Glycopeptides** Vancomycin Teicoplanin	
Tetracyclines	Tetracycline Minocycline	Protein synthesis inhibitors
Macrolides	Erythromycin Azithromycin	
Aminoglycosides	Gentamicin Tobramycin	
Sulfonamides	Sulfadiazine Sulfisoxazole	Folic acid synthesis inhibitors
Trimethoprim	Trimethoprim Sulfamethoxazole	

Continued Table 2.

Class	**Family*** / examples	**Mechanism**
Nitrofurantoin	Nitrofurantoin	DNA damage
Metronidazol	Metronidazol	DNA synthesis inhibitors
Quinolones	Ciprofloxacin Ofloxacin Norfloxacin	

*Families of antibiotics are written in bold

1.7. Development of resistance to antimicrobial agents

A few decades after the introduction of antibiotics into clinical practice, resistance by pathogenic bacteria has become a major health concern. Indeed, while in the mid 1970s infectious diseases were considered virtually conquered [30], actually many gram positive bacteria and gram negative opportunistic pathogens are becoming resistant to virtually every clinically available drug [31, 32]. The use of antimicrobial drugs for prophylactic or therapeutic purposes in human and veterinary or for agricultural purposes, has provided the selective pressure favouring the survival and spread of resistant organisms. *Staphylococcus aureus,* for instance, a virulent pathogen that is responsible for a wide range of infections including pimples, pneumonia, osteomyelitis, endocarditis and bacteremia, has developed resistance to most classes of antibiotics. Methicillin-resistant *S. aureus* (MRSA) strains appeared in the hospital environment after introduction of the semi-synthetic penicillin and methicillin, being vancomycin the last chance for MRSA treatment [33]. Certainly, Vancomycin is the last tool for the treatment of the infections caused by the resistant gram

positive microorganisms. Indeed, vancomycin resistance is difficult to acquire because is a complex system involving up to 7 genes. However vancomycin-intermediately-sensitive *S. aureus* were first isolated in 1997 in Japan [34] and later in other countries [35]. In fact, vancomycin-resistant clinical isolates have been recently reported [36-38]. Thus currently, no antibiotic class is effective against multi-resistant *S. aureus* infections and new antibiotics or alternative chemotherapeutic strategies are urgently needed.

Enterococci are responsible for urinary tract, wound, intraabdominal, and pelvic infections [39]. The increase of vancomycin-resistant enterococci (VRE) as important agents of nosocomial infections is cause of great concern [40, 41]. It is thought that a selective pressure favouring the survival and spread of VRE was the consequence of the use of antibiotics in food and agricultural practices [42]. Actually, there are no effective antibiotics currently available for such organisms. Even worst, vancomycin-resistance is often associated with multiple-drug resistance [40, 41, 43, 44].

Another cause of great concern is the gram-negative antibiotic-resistant opportunistic pathogens. These bacteria, like *Pseudomonas aeruginosa*, are common environmental organisms, which act as opportunistic pathogens in clinical cases where the defence system of the patient is compromised [45]. For instance, over 80 % of cystic fibrosis (CF) patients become chronically infected with *P. aeruginosa*. In addition, other intrinsically antibiotic-resistant organisms such as *Burkholderia cepacia* [46] and *Stenotrophomonas maltophilia* [47] are emerging as opportunistic pathogens. Interestingly, changes in the bacterial phenotype have been observed concomitant with the appearance or increase of antibiotic resistance. Indeed, in CF infections, initially, strains are non mucoid, but over time a mucoid population showing slow growth phenotype with a increased capability to form

biofilms, the small colony variants (SCV), develops [48]. This ability is considered a major virulence trait because the bacteria are protected from adverse environmental conditions as well as from biological and chemical antibacterial agents [49]. Thus, new therapeutic drugs and/or approaches are needed to improve the management of these diseases and overcome these problems [50, 51].

In conclusion, the appearance of multiresistant pathogenic strains has caused a therapeutic problem of enormous proportions. For instance, they cause substantial morbidity and mortality specially among the elderly and immunocompromised patients. In response, there is a renewed interest in discovering novel classes of antibiotics that have different mechanisms of action [23, 39].

2. GOAL OF THE STUDY

The main aim of this study was the discovery secondary metabolites with antibiotic activity against microbial pathogens resistant to antibiotics currently used in the clinical practice.

To achieve this goal the following specific objectives were pursue:

- Identification of active secondary metabolites contained in MeOH extracts produced by 2048 isolates obtained from a wide variety of environmental sources.

- Optimisation of cultivation parameters in order to improve the yields and increase the number of secondary metabolites available from different microbial sources.

- Chemical characterisation of secondary metabolites with high biological activities .

- Purification and structure elucidation of novel active secondary metabolites.

- Chemical and biological characterisation of the new compound, 7-*O*-malonyl macrolactin A, showing activity against bacterial pathogens.

3. MATERIALS AND METHODS

3.1. Microorganisms

3.1.1. Isolation, origin and maintenance of producing microbial strains

Bacterial strains were obtained from a variety of environmental sources from different geographical areas (Kenya, Indonesia, Italy, Vietnam, Mexico and Germany). The strains were isolated in the Department of Enviromental Microbiology, German research center for Biotechnology - GBF (Braunschweig, Germany) in the course of its studies on the structure and function of natural microbial communities. Particularly were included into the study, the organisms that inhabit extreme or poorly explore areas, like hypersaline or volcanic environments. As an alternative, environmental samples were used as source of microorganisms used in this study. For their isolation 10 –20 mg of the samples were incubated in 50 ml of phosphate-buffered saline (PBS) 1X solution at room temperature with shaking for 1h. 100 µl of the PBS containing the sample were platted in petri dishes with LB agar medium and incubated over night at 30°C. The isolated colonies were cultivated in separated plates and stored in liquid LB with 20 % of glycerol at -80°C. Altogether, 2048 environmental strains were tested for biologically active secondary metabolites. Strains showing biological activities were identified using 16S rRNA sequence analysis.

3.1.2. Target microorganisms

Several target microorganisms were used to assess the antimicrobial activity of the MeOH extracts obtained from environmental isolates. Bacterial target strains were growth over night at 37°C in Luria Broth (LB) except for *S. aureus*, *E. faecalis* and *E. faecium*, which

were grown on Columbia blood agar. Yeast and fungi strains were growth in M90 and Potato Dextrosa agar media, respectively. The assessment of the antimicrobial activities was performed in Mueller-Hinton (MH) broth and agar (Difco). The species, origin and sources of the target microorganisms are listed in Table 3.

Table 3. Microorganisms used as target to assess the antibacterial activity of the produced microbial extracts.

Strains	Origin	Remarks
Staphylococcus aureus	GBF Collection	
Staphylococcus aureus	DSM 2569	
Staphylococcus aureus	DSM 1104	
Staphylococcus aureus	3 clinical isolates	MRSA
Enterococcus faecalis	ATCC 29212	
Enterococcus faecalis	E305 clinical isolate	VRAS
Enterococcus faecium	E315 clinical isolate	VRAR
Escherichia coli	DSM 498	
Pseudomonas aeruginosa	DSM 1117	
Pseudomonas aeruginosa	3 isolates from CF patients	WT- antibiotic multiresistant
Pseudomonas aeruginosa	3 isolates from CF patients	SCV- antibiotic multiresistant
Pseudomonas aeruginosa	12 isolates from CF patients	NM - antibiotic multiresistant
Burkholderia cepacia	3 isolates from CF patients	WT - antibiotic multiresistant
Burkholderia cepacia	3 isolates from CF patients	SCV- antibiotic multiresistant
Stenotrophomonas maltophilia	15 clinical isolates	antibiotic multiresistant
Candida albicans	DSM 11225	

Continued Tabla 3.

Strains	Origin	Remarks
Candida glabrata	DSM 11226	
Candida tropicalis	DSM 5991	
Candida parapsilosis	DSM 5784	
Candida krusei	DSM 6128	
Pichia anomala	DSM 70263	
Metschnikowia pulcherrina	DSM 70321	
Schizosaccharomyces pombe	Tü 501	
Botrytis cinerea	DSM 877	
Fusarium fujikuroi	DSM 893	
Penicillium digitatum	DSM 62840	

MRSA: methicillin resistant *Staphylococcus aureus*
VRAS: vancomycin resistant-ampicillin sensitive
VRAR: vancomycin resistant-ampicillin resistant
WT: wild type
SCV: Small colony variants
CF: Cystic fibrosis
NM: Non mucoid
All clinical isolates from CF patients and *S. maltophilia* were kindly provided by Susanne Häußler (Department of Medical Microbiology, Medizinische Hochschule Hannover), except for *P. aeruginosa* non-mucoid strains provided by Prof. Niels Høiby (Department of Paediatrics, Danish Cystic Fibrosis Center, Rigshospitalet, Copenhagen O, Denmark).

3.2. Antibiotics

Several commercial antibiotic discs (BioMerieux and Rosco) were used to evaluate and control the pattern of antibiotic sensitivity of the different target strains. Erythromycin (ERY), Vancomycin (VAN), Ampicillin (AMP), Gentamicin (GEN) and Miconazole

(MCZ) were obtained from Sigma-Aldrich. Stock solutions were freshly prepared at 10 mg/ml in sterile distilled water, except for erythromycin, which was prepared in ethanol.

3.3. Production and extraction of secondary metabolites

Different solid and liquid media were used to cultivate the environmental strains. The medium selected for each strain was chosen according their origin of the isolate during the preparation of the first extract for the screening (see media in Appendix). Culture extracts were obtained as described by Sasse *et al* [52]. Flasks containing 200 ml of medium were inoculated with 500 µl of microbial preculture. At the same time, an adsorbent resin amberlite XAD-16 (Rohm & Haas, Germany) was added (4 %) and the flasks were incubated at 30°C and 120 rpm, for 7 days. The resin was recovered from the culture broth by decantation, transferred into a column and washed with 50 % aqueous MeOH. Absorbed products were eluted with 100 % MeOH, concentrated by rotavaporation and finally resuspended in 2 ml of MeOH. MeOH extracts were stored at –80 or –20°C.

3.3.1. Extraction of macrolactin compounds

Specifically, for the extraction of the macrolactin compounds, MeOH extracts were obtained from 7 day cultures of *B. subtilis* MIK-SM 2586 in OM medium. After MeOH evaporation, the remaining aqueous mixture was extracted 4 times with ethyl acetate. Evaporation of the solvent under reduced pressure provided approximately 300 mg of oily residue from a total of 4 L of culture. This was resuspended in MeOH and partitioned four times with the same volume of n-heptane to remove the more lipophilic products/contaminations.

3.4. Biological tests

3.4.1. In vitro antibacterial activities

The in vitro antibacterial activities were evaluated using a disc diffusion test on solid medium and a microdilution test in liquid medium. For the agar diffusion test, sterile discs (Schleicher & Schuell, Germany) impregnated with 10 µl of MeOH extract or a solution of purified macrolactins (50 µg final macrolactin concentration on the disc) was placed on the dried surface of MH agar plates. The plates were previously inoculated with a standardized suspension (10^5 CFU/ml) of the different target microorganisms. The suspensions were prepared from a one-day culture in LB liquid medium. After overnight incubation at 30°C, the diameter of the resultant zones of inhibition of growth around the disc was measured.

Microdilution tests were performed using MH broth. Microtiter plates containing 50 µl of twofold serial dilutions of each antimicrobial agent or MeOH extract per well were inoculated with 50 µl of a bacterial suspension to yield the appropriate density (1 to 5 x 10^5 CFU/ml). In parallel MeOH dilutions and MH broth alone were used to control the microbial growth. The lower concentration of antibiotic or the higher dilution of MeOH extract that were able to inhibit the target microbial growth was recorded after 18 h of incubation at 37°C.

3.4.2. Antifungal and antiyeast activities

Antifungal and antiyeast activities were evaluated using an agar diffusion test, following a similar procedure as for antibacterial activity (Numeral 3.4.1.). These experiments were carried out by Olga Golyshina (Division of Microbiology, GBF). The media used were M90, for antifungal and MH for antiyeast assays. The time of incubation was variable (1-3

days) depending on the rate of the growth of the fungi or yeast.

3.4.3. Insecticidal activity

Insecticidal activity of the MeOH extracts was tested against four insects targets belonging to the order Lepdidoptera (*Spodoptera frugiperda*), Diptera (*Musca domestica* and *Anopheles albimanus*) and Coleoptera (*Tenebrio molitor*). The experiments were performed in the "Corporacion para Investigaciones biologicas" (Medellin, Colombia).

3.4.3.1. Insecticidal activity against mosquitoes

Five first instar mosquito larvae of *Anopheles albimanus* were placed in 24 well plates with 960 µl of sterile water, to which 40 µl of the extract were added. Water, and MeOH/water were used as controls. Mortality of *A. albimanus* larvae was scored after 24 h of incubation at 30°C.

3.4.3.2. Insecticidal activity against *Spodoptera frugiperda*

A total of 40 µl of the MeOH extract were added to the surface of a beans based diet served in disposable cups. After 4 hours, five first instar *S. frugiperda* larvae were placed on the surface of the diet. Sterile phosphate-buffered saline (PBS) and MeOH were used as controls. Mortality of *S. frugiperda* larvae was scored after 72 h.

3.4.3.3. Insecticidal activity against *Musca domestica*

The activity of the MeOH extracts was evaluated using the filter paper technique. Filter paper was placed in a petri dish and moistened with 40 µl of MeOH extract diluted in 360 µl of distilled water. Three *M. domestica* larvae were placed in each dish. Activity was

recorded after 72, 144 and 192h at 17°C by scoring larval mortality, teratological effects and adult emergence. Water and MeOH / water were used as controls.

3.4.3.4. Insecticidl activity against *Tenebrio molitor*

10-20 mg artificial diet for *T. molitor* (corn meal 95 %, yeast 5 %) were moistened with 40 µl of each MeOH extract dissolved in 1 ml of sterile water and lyophilised over night. The mix was placed in 24 wells plates. Two neonate larvae of *T. molitor* were transferred to each well. The bioassay was observed after 7 days at room temperature. Water and MeOH / water were used as controls.

3.4.4. Cytotoxic activity

The eukaryotic cell cytotoxicity assay is used for evaluating the potential toxic properties of the compounds. Cytotoxicity includes changes in the normal cellular growth or expression which alters the ability of the cell to reproduce itself or maintain its normal tissue specific function. The cell proliferation process can be also promoted or retarded. Cytotoxicity assays were performed by Dr. Gabriella Molinari (Division of Microbiology, GBF). HeLa human cervical carcinoma cells and L929 mouse fibroblast were cultured in DMEM (Gibco) low and high glucose, respectively. These media were supplemented with 10 % (v/v) fetal bovine serum (Gibco) and maintained at 37 °C with 5 % CO_2. Cells were removed from monolayer stock cultures with trypsin, with and without EDTA (Gibco), for HeLa and L929, respectively. The cells were counted, diluted to obtain 10^4 cells/ml and inoculated in microtiter plates alone and in the presence of serial dilutions of the MeOH extracts or purified compounds. MeOH was used as control. Morphological changes in cells were evaluated by phase-contrast microscopy after 1, 2 and 5 days of incubation.

Finally, after 5 days, cell counts were obtained using the CyQUANT cell proliferation assay (Molecular Probes), a highly sensitive, fluorescence-based microplate assay for determining numbers of cultured cells [53]. The assay employs CyQUANT dye, which produces a large fluorescence enhancement upon binding to cellular nucleic acids that can be measured using fluorescein excitation. The fluorescence emission of the dye-nucleic acid complexes correlated linearly with the cell number. [53]. The assay was performed according to manufacturer instructions measuring fluorescence with an excitation of 480 nm and emission of 520 nm with a fluorometric plate reader (Titertex Fluoroskan II).

3.4.5. Minimal Inhibitory Concentrations of 7-*O*-malonyl macrolactin A

The standard twofold serial microdilution method described by the National Committee for Clinical Laboratory Standards (NCCLS) [54] was performed for the assessment of the minimal inhibitory concentrations (MICs). For this test 96 wells plates containing 100 µl per well of MH broth with purified macrolactins or commercial antibiotics were used. In parallel MeOH dilutions and MH broth alone were used as microbial growth controls. Each well was inoculated with 10^5 cells/ml of the target microorganisms. The MIC was defined as the lowest antibiotic concentration that completely prevented visible growth after incubation at 37°C for 20h.

3.4.6. Kinetics of growth at sub-Minimal Inhibitory Concentrations of 7-*O*-malonyl macrolactin A

During the MIC determination, a strong reduction of microbial growth, but not full inhibition, was observed at sub-MICs of 7-O-malonyl macrolactin A (7-O-m). Thus, growth kinetics of the selected target microorganisms was evaluated under these

concentrations. Fresh MH broth with appropriate 7-O-m concentration was inoculated to about 10^6 CFU/ml from overnight cultures. The test tubes were incubated at 37°C and 150 rpm. Aliquots of the culture were removed at 0, 1, 2, 4, 6, 8 and 24 hours, and dilutions were plated in LB agar medium using a spiral plater (Spiral Biotech). After 24 hours incubation at 37°C, colony counts were determined using a laser colony counter (CASBA 4, Spiral Biotech). Finally, growth profiles were constructed.

3.4.7. Post-Antibiotic effect measurements of 7-*O*-malonyl macrolactin A

Post-antibiotic effect (PAE) is a term used to describe suppression of bacterial growth that persist after brief exposure of organisms to antimicrobials. PAE measurements at sub-MICs were determined with the method of Craig and Gudmundsson [55]. To induce PAE, 7-O-m was added to tubes containing MH broth to give a final concentration 4 times the sub-MIC. These tubes were inoculated with overnight cultures of MRSA3 and VRAR *E. faecium* at 10^6 CFU/ml and incubated at 37°C. After 1h the samples were diluted 1:1000 in drug free MH broth warmed at 37°C in order to remove the antibiotic. Test tubes without antibiotic were used as controls. The treated and control cultures were then incubated at 37°C. Viability counts were determined before exposure and immediately after dilution (0 h) and then 1, 2, 4 and 6 h by plating in MH agar medium using a spiral plater (Spiral Biotech) and incubated for 24 hours at 37°C. Colony counts (CFU/ml) were determined using a laser colony counter (CASBA 4, Spiral Biotech).

The PAE was defined by the equation: PAE = T - C, where T is the time required for viability counts of an antibiotic exposed culture to increase by 1 $\log_{10}$ above the counts observed immediately after dilution and C is the corresponding time for the growth control [43].

3.4.8. Transmission electron microscopy

For transmission electron microscopy (TEM), 7-O-m was added to tubes containing MH broth to give a final concentration equal to 4 times the sub-MIC. The tubes were inoculated with overnight cultures of MRSA3 and VRAR *E. faecium* at 10^6 CFU/ml and incubated at 37°C for 4 h. Tubes without antibiotic were used as controls. The cells were harvested by centrifugation at 12000 rpm and fixed with 1 % (v/v) glutardialdehyde in phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7±2). After washing in 0±1x PBS, the cells were post-fixed with 1 % OsO (w/v) in 0±1 M sodium cacodylate, pH 7±0, overnight at 4°C. Cells were immobilized in 2 % (w/v) Difco agar in PBS. Dehydration in an acetone series (30, 50, 70, 90, 100 and 100 %, v/v) was done at ambient temperature, requiring 20 min for each dehydration step. Infiltration with an embedding resin/acetone mixture (1:1, v/v) was done at ambient temperature overnight, followed by pure resin infiltration overnight and finally for 4 h [56]. Probes were transferred into a gelatine capsule and filled with resin monomer. Polymerization was done for 8 h at 70 °C. Ultrathin sections were cut with a diamond knife using the Leica Ultracut ultramicrotome and were picked up with Formvar-coated grids (300 mesh, Cu). Sections were poststained for 10 min with 3 % (w/v) aqueous uranyl acetate and for 5 min with 0±5 % (w/v) lead citrate as described by Reynolds [57]. The samples were examined using a transmission electron microscope (Zeiss CEM 902, conventional mode, 30 lm objective aperture, 80 kV acceleration voltage). Electron micrographs were taken digitally with a high resolution CCD camera (Proscam).

3.5. Identification of the active compounds

Different strategies were used for determining the chemical nature of the active substances contained in the MeOH extracts with biological activities.

3.5.1. Thin Layer Chromatography for determination of the active substance

Thin layer chromatography (TLC) was carried out using TLC aluminium sheets silica gel 60 F 254 (Merck 05554) or TLC plates SIL 20 UV 254 (Macherey-Nagel). Separation of the compounds present in the active MeOH extracts was achieved by applying 3 µl of the extract to a silica gel TLC plate, which afterwards, was immersed in the appropriate solvent (Table 4). Afterwards, the plate was covered with MH agar, inoculated with the target strain and incubated over night at 30°C. Inhibition zones resulted in the area in which biologically active compounds diffused. These inhibition zones were used to define the areas to scrape out of a parallel TLC carried out in the same conditions. The active compound was eluted with MeOH.

Table 4. Solvents used for TLC.

Mixture	**Concentrations**
CH_2Cl_2: MeOH	80:20 to 90:10
Ethylacetate:MeOH:H_2O	85:15:1
$CHCl_3$:MeOH:NH_3^+	65:25:8 to 65:25:12
MeOH:H_2O	80:20

TLC of the MeOH extracts was carried out in four solvents depending on the chemical

properties of the active compound. On the basis of the results of the first running, either different concentrations of the same solvent or other solvents were employed for efficiently separation and isolation of active compounds.

3.5.2. Silica gel column chromatography for determination of the active substance

Active compounds were purified by column chromatography on silica gel with the appropriate solvent. One ml of extract was placed in a glass column of 50 cm previously equilibrated and eluted with 3 volumes of 4 different concentrations of the same solvent. Around 50 fractions of 5 ml each were collected and their antimicrobial activity tested. The active fractions were identified and concentrated to be further characterised.

3.5.3. High performance liquid chromatography for determination of the active substance

High performance liquid chromatography (HPLC) was carried out using a Waters HPLC system 2690 equipped with a pump and a Water photodiode array detector (PDA 996) with variable wavelength detector (220-800nm). The column used was a Nucleosil 100-5 C18 column (250/4 mm, Macherey-Nagel). The solvents used were A: 0.5 % Formic acid/H_20 and B: 0.5 % Formic acid/Acetonitrile. Gradient 10 % B 0 min; 10 % B 5 min; 20 % B 10 min; 30% B 15 min; 45 % B 20 min; 60 % B 25 min; 85 % B 28 min; 100 % B 30 min; 10 % B 32 min; the flow rate was 1.0 ml/min. All solvents were degassed prior to use. 50 µl of the MeOH extract were injected and 60 fractions (every 30 seconds) were collected with a Waters fraction collector. The biological activities of each fraction were compared with the activity of the MeOH extract using the agar diffusion test. Fractions showing biological activity were identified in the chromatogram and the corresponding UV spectra analysed.

3.5.4. Identification of the antimicrobial substance by HPLC-UV-MS

HPLC-UV-MS was analysed by Dr. Rolf Jansen (Department of Natural products, GBF) using a Hewlett Packard HPLC system (HP 1100) equipped with a DAD diode array UV detector. Separation was achieved with a Nucleosil 120-5 RP C18 column (125/2mm, Macherey-Nagel) coupled to a PE Sciex API 2000 LC/MS/MS system with an ACI device. The temperature used was 40°C. The active substances were identified using the masses and UV spectra in comparison with the following compound databases:

"Dictionary of Natural Products" database (Chapman and Hall/CRC) on CD rom, Antibase 2000 (VCH Wiley), and CrossFire Beilstein databases (MDL) (on-line).

3.6. Spectrometric analyses and structure determination of the macrolactins

3.6.1. Purification of the macrolactins using high performance liquid chromatography (HPLC) and LH-20 chromatography

MeOH extracts and fractions were analysed by RP-HPLC using a Hewlett Packard 1090 Series II HPLC system equipped with a gradient pump system, an UV diode array detector at 220-600 nm and an external light scattering detector (PL ELS-1000; Polymer Laboratories); Separation was achieved with a Nucleosil 100-5 C18 column (125/2 mm, Macherey-Nagel); the used solvents were A: 0.5 % acetic acid/H_20 and solvent B: 0.5 % acetic acid/MeOH; and the following gradient was used at a flow rate of 0.3 ml/min: 52 % B 0 min; 60 % B 24 min; 100 % B 26 min; 100 % B 31min; 52 % B 32 min; 52 % B 35 min; 3 µl of MeOH extract were injected.

The macrolactins were separated by preparative RP-HPLC, using a Nucleosil column 100-7 C18 (250/21mm, Macherey-Nagel); The solvents used were A: 0.5 % acetic acid/MeOH 51 % and B: 0.5 % acetic acid/MeOH 56 %. 100 % A 0 min; 100 % B 60 min; 100 % B 35

min; flow rate 20 ml/min; the amount of extract injected was 40-60 mg of extract in 0.2 ml MeOH; UV detection at 280 nm.

Each macrolactin was further purified by LH-20 chromatography, using a glass column 760/25 mm; the solvent used was MeOH/dichloromethane (1:1); flow rate 5 ml/min. 5-7 mg of the compound was injected.

3.6.2. HPLC- UV-MS and MS-MS analysis

HPLC-UV-MS was performed in the Department of Structure Biology, GBF, with the same procedure as in numeral 3.5.4. MS data were obtained on a MAT 95 mass spectrometer in EI and DCI mode (Finnigan).

3.6.3. Nuclear magnetic resonance (NMR)

NMR analyses were performed in the Department of Structure Biology, GBF. For NMR spectroscopy the samples were dissolved in 99,95 % MeOH-d_4, chloroform-d_3 or dichloromethane-d_2; the data were obtained with a WM-600 spectrometer (Bruker).

3.6.4. Optical Rotation and UV spectrum

Optical rotation and UV spectrum were measured in UV MeOH (Merck) on a Polarimeter MC 241 (Perkin Elmer) (d = 10 cm) and a UV-2102 PC UV-VIS Scanning Spectrophotometer (Shimadzu).

3.7. Optimisation of secondary metabolite production

The optimal medium and conditions of growth for secondary metabolite production was investigated. Media (appendix media used) were modified in order to establish the optimal

conditions for production of the active secondary metabolites or in some cases to recover the activity. Different compounds for promoting the production of secondary metabolites were also added (Mg^{+2}, Zn^{+}, Mn^{+2}, Fe^{+}, and DMSO) and their influence in the bacterial growth and production of the active substance was studied and particularly, with the Cooper media composition was modified in order to study the influence of each component (See results). The evaluation of the efficiency and diversity of secondary metabolite production was performed analysing the HPLC spectra of the obtained MeOH extract, produced biomass and antibacterial activities of the different MeOH extracts obtained from the same producer microorganism. Biomass production was determined by the dry weight technique. For this purpose 1.5 ml of the cultures were placed in pre weighed microtubes, centrifuged at 12000 rpm for 10 min. After discarding the supernatant, obtained pellets were dried under vacuum conditions for 24 h. Afterwards, the tubes were weighted again and the biomass was calculated from the weight difference. The amount of macrolactin in the MeOH extract obtained from the different media was measured by diffusion agar test using *Staphylococcus. aureus* as assay organism. Concentration of macrolactins was estimated from a calibration curve made using pure macrolactins. In addition, chromatograms of the MeOH extracts were obtained to assure that the increase of inhibition observed in the diffusion test was due to the increase of macrolactins production.

3.8. Optimisation of macrolactin production

3.8.1. Selection of optimal media for macrolactin production

20 media with different composition were tested for production of macrolactins by *B. subtilis*. Studied medium were: Xgen, *Bacillus pumilus* medium (Bpm), EM, Cooper,

GEN, SMM, Landy, CA, St-1, St-3, MIC 10, KMA, KMB, F1, LB, TSYEM, Seed, M61, R2A and OM (See media appendix). The strain was grown in the different media using the already mentioned procedure (Numeral 3.3). Tested parameters biomass and macrolactins production were measure as in numeral 3.7.

3.8.2. Addition of compounds for promoting secondary metabolite production

After selecting the best media for macrolactin production (Numeral 3.8.1), it was modified by the addition of different compounds for promoting the production of secondary metabolites (Mg^{+2}, Zn^{+}, Mn^{+2}, Fe^{+}, and DMSO). The effect of such additions on the biomass and macrolactin production was studied as previously described (Numeral 3.8.1).

3.8.3. Influence of the media composition in the production of macrolactins

The influence of each component of the medium selected for macrolactin production (Numeral 3.8.1) on the bacterial growth and production of the active substance was analysed. Modifications to the standard medium are presented in results. The evaluation of the biomass and macrolactins production was analysed as mentioned above (Numeral 3.7.3).

3.8.4. Effect of pH in macrolactin production

Once the optimal composition of the media was established, the effect of the pH on both biomass and antibiotic production was tested. The tested pH conditions were 5.0, 7.0, 9.0 and 10.0. Biomass and antibiotic production was quantified as described above.

3.8.5. Macrolactin production during bacterial growth

The macrolactins production on the optimised medium was monitored during 9 days of bacterial cultivation. For this purpose, 10 different parallel cultures were inoculated and cultured at the standard conditions (Numeral 3.3). Every day, one culture flask was withdrawn and a MeOH extract prepared from it. Biomass and macrolactin production were determined as above described (Numeral 3.8.3).

3.9. Two-dimensional gel electrophoresis

3.9.1. Sample preparation

100 ml of MH broth were inoculated at 10^7 CFU/ml with overnight cultures of methicillin resistant *Staphylococcus aureus* (MRSA3) and incubated at 37°C for 16 h in presence of 4 μg/ml of 7-O-m (sub-MIC). Flasks without antibiotic were used as controls. The cells were harvested by centrifugation at 12000 rpm and stored at –70°C. Cell material (10 mg wet weight) was resuspended in 1 ml of reswelling solution for electrofocusing (IEF) (7.4 M of urea, 2 M thiourea, 4 % CHAPS, 30 mM dithiotreitol, 20 mM Trizma base, protease inhibitor cocktail [one table of Mini(tm) Complete per 20 ml solution; Roche]). Afterwards, the suspension was sonicated 6 times for 30 s (1 s repeating duty cycle, Labsonic U; Braun). Protein concentrations were determined by the procedure of Bradford [58].

3.9.2. Electrophoresis

Two-dimensional gel electrophoresis was carried out according to the method of Görg [59]. About 300 μg of protein was applied to dry pH 3-10 IPG ReadyStrips (Bio-Rad). IEF was performed in a protean 150 kVh in total. The strips were then transferred to 1.5-mm-

thick 12-15 % gradient SDS-polyacrylamide gels and developed overnight in the isoDalt system from Amersham Pharmacia Biotech. The gels were stained using colloidal Coomassie R-250, according to the method of Blum *et al*. [60]. Differential protein expression was visualized with the Z3 image analysis software (Compugen; http://www.2dgels.com). Protein spots of interest were excised and the samples prepared for MALDI-TOF according to Wissing *et al* [61].

4. RESULTS

The main purpose of this study was to discover novel biologically active secondary metabolites. Initially, it was necessary to obtain a great variety of strains from extreme environments around the world to access a wide diversity of microbes. The strains were evaluated for the production of secondary metabolites and subsequently, the ones producing secondary metabolites with interesting activities were further investigated to isolate and chemically characterise the substances responsible for this biological activity.

4.1. Microorganisms producers of secondary metabolites with biological activities

Bacteria, fungi, yeast, eukaryotic cells and insects were used as targets to evaluate the biological activity of 2048 MeOH extracts produced by the same number of bacterial strains isolated from different geographical areas. A total of 137 (6.7 %) strains (Figure 2), belonging to 25 genera (Figure 3), produced secondary metabolites with biological activity against one or more targets (Figure 4).

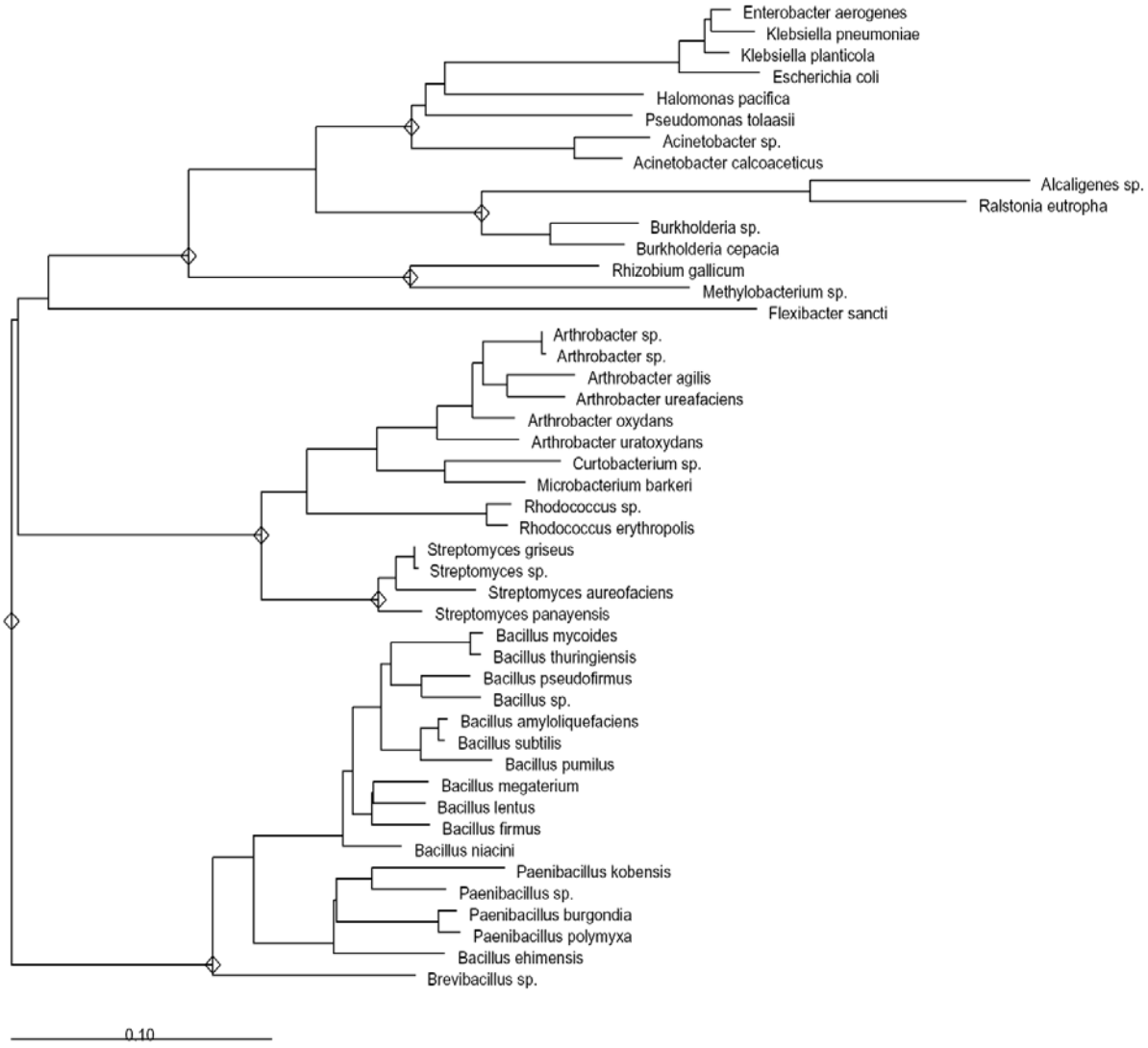
Of the 137 microbial active extracts, 81 (59.1 %) showed antibacterial activity. Among them, 27 showed activity only against bacteria, 44 against bacteria and fungi/yeast, 12 against bacteria and insects, and 16 showed antibacterial and cytotoxic activities. Antiyeast and antifungal activities were detected in 79 from the active 137 MeOH extracts (57.6 %). Among them, 67 extracts were active only against fungi, 10 against both targets and 2 were active exclusively against yeast. However, it is important to mention that from the originally 79 strains producing antifungal and antiyeast compounds, 74 of them either lost the ability to produce active secondary metabolites after the storage (low stability) or produced very low amount of active compounds. Therefore, only 5 antifungal and antiyeast

MeOH extracts were further analysed for chemical identification.

From the 2048 MeOH extracts, 1536 were tested for cytotoxicity and cell proliferation effects on eukariotic cells (HeLa and L929). A total of 120 extracts (7.8%) were active by either affecting morphology and cell proliferation (71 extracts) or cytotoxic effect (49 extracts). On the basis of these cytotoxic results, a group of 80 active extracts were selected for testing their activity against 4 different insect targets. A strong insecticidal activity against *Anopheles albimanus* and *Musca domestica* was detected with 28 extracts (35 %). Specifically, 20 extracts showed larvicidal activity against first instar larvae of *A. albimanus* and 9 against *M. domestica*. Noteworthy, when mortality of *M. domestica* larvae was not observed, retardation of the larval development and/or absence of adult emergency were frequently observed. Finally, non of the extracts showed activity against the other insect targets (*Spodoptera frugiperda* and *Tenebrio molitor*).

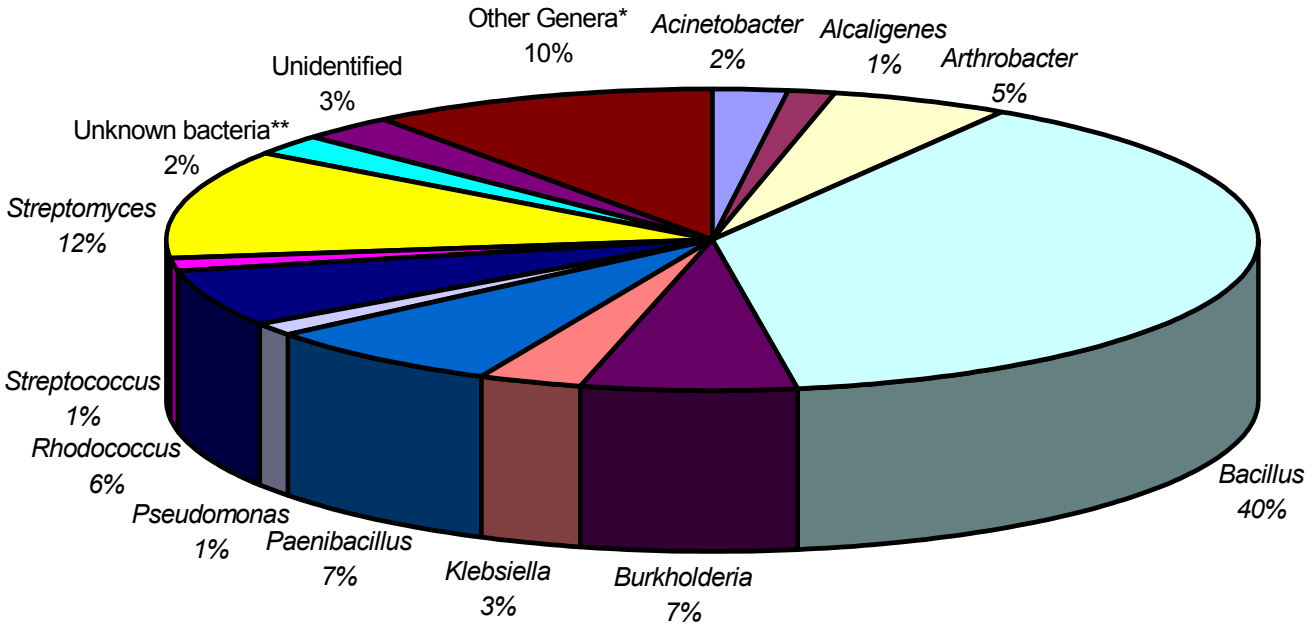
The tested microbial extracts were actually a mixture of secondary metabolites. However, our aim was to isolate and identify the specific compounds conferring this biological activity. Therefore, the extracts with high biological activities against the different target microorganisms were further analysed.

Figure 2. Phylogenetic relationships of the environmental isolates producing active secondary metabolites.



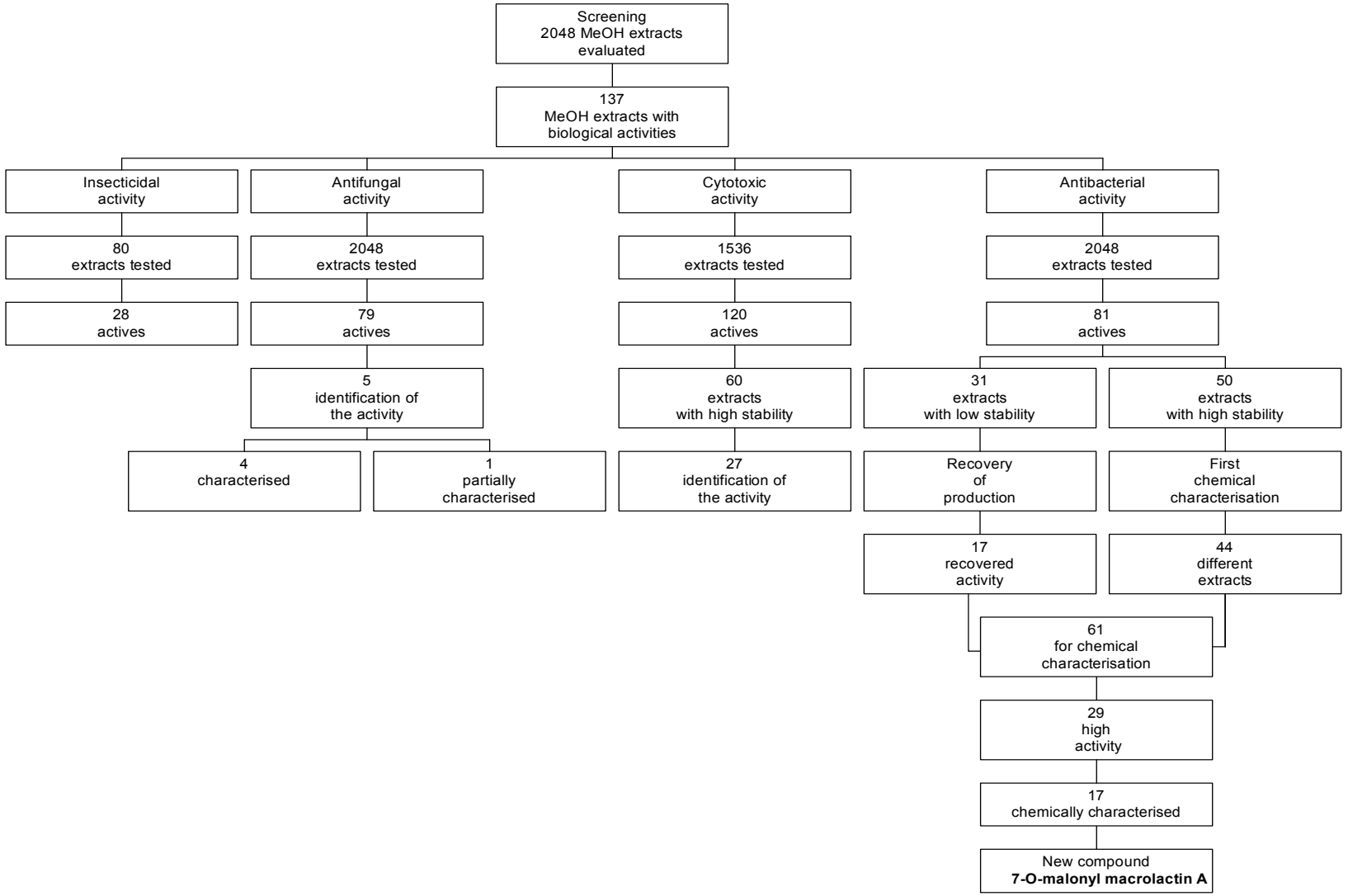
The isolates producing active secondary metabolites were identified using 16S rRNA sequence analysis.
The score bar represents 1 nucleotide substitution per 100 nucleotides.

Figure 3. Taxonomical classification of the 137 strains producing active secondary metabolites.



* Different genera represented by only 1 microorganism/each
** Comparative 16S rRNA sequence analysis of these strains was related to uncultivated bacteria

Figure 4. Synopsis of the screening for active secondary metabolites and biological activities of microbial extracts obtained during the study.



4.2. Optimisation of secondary metabolite production in strains showing low stability

A total of 81 strains produced antibacterial activity after their isolation. However, 31 strains lost the capacity of producing antibacterial compounds when a second MeOH extract was prepared from the stored glycerol stock. The fact that some strains lose the capacity of producing secondary metabolites with antimicrobial activity after already short storage periods is not unusual, since, it have been reported that most of the microbial strains are active producers of secondary metabolites only when they are freshly isolated [5]. The fact that the strains lost the capacity to produce active secondary metabolites can be attributed to unfulfilled nutritional needs or opposite, appropriate cultivation conditions which thus do not stress the producer strain which is required for secondary metabolite production [24.]. To recover the capacity of production of active substances a series of media used for secondary metabolite production were tested. Media for each microbial producer were selected after an intensive literature search and are summarised in Table 5. The evaluation of the stability of secondary metabolite production was performed comparing the biological activities of the different MeOH extracts obtained after cultivation in the different media. Most of the bacterial strains grew in at least 2 of the tested media. However, regaining the capacity to produce biologically active compounds was only achieved in 17 of the 31 tested strains. Therefore, it is clear that optimal growth can not be correlated with production of active secondary metabolites. Interestingly, with *B. pumilus* (strains MIK-SM 1014 and 2329) growth of the strain and production of the active compounds in the different media followed different patterns, even when both isolates belong to the same specie. Similar results were observed with the strains MIK-SM 1242, 1244 and 2249 of *B. laterosporus*. As it can be concluded, optimal conditions for

secondary metabolite production seems to be strain related and not even related to strains belonging to the same genus or specie (Table 5).

Table 5. Optimisation of secondary metabolite production in strains showing low stability: effect of cultivation media in antimicrobial activity obtained.

MIK-SM number	**Microorganisms**	**Media**	**Microbial Growth**	**Antimicrobial Activity**
1225	*Deleya pacifica*	**M61**	+	+
		ME	+	-
		M284	+	-
		M109	+	-
		EM	-	-
		LB	-	-
		Cooper	-	-
		PDB	-	-
		DSM	-	-
		OMLP	-	-
		Landy	-	-
		Xgen	-	-
2160	*Bacillus thuringiensis*	**OM**	+	+
		GEN	+	-
		Xgen	+	-
		Bpm	+	-
		Cooper	+	+
380	*Bacillus pseudofirmus*	**M 61**	+	+
		SMM	-	
		Landy	-	
		Xgen	-	
		Cooper/Mn	+	-
		ANA	+	-
382	*Bacillus agaradhaerens*	**M61**	+	+
		ANA	+	-
		Landy	-	
		Xgen	-	
		Cooper/Mn	-	
1014	*Bacillus pumilus*	**EM**	+	+
		Cooper	+	+
		OMLP	+	+
		DSM	+	+
		Landy	+	+
		Xgen	+	+
		Bpm	+	+

Continued Table 5.

MIK-SM number	Microorganisms	Media	Microbial Growth	Antimicrobial Activity
2329	*Bacillus pumilus*	**R2A** Bpm Landy Xgen Cooper/Mn	+ - + + +/-	+ - + -
1223	*Bacillus subtilis*	**M61** XGen Landy DSM Cooper/Mn NBG	+ + + + + +	- - - - - -
1237	*Bacillus sp.*	**M61** Cooper EM	+ + +	+ + -
1241	*Bacillus lentus*	**M61** CA Landy Xgen Cooper/Mn Cooper	+ + - + - +	+ - - +
1242	*Bacillus laterosporus*	**M61** Cooper Xgen Bpm OM Seed Medium OMLP EM	+ + + + + + + +	+ + - + + - - +
1244	*Bacillus laterosporus*	**M61** Cooper EM	+ + +	+ + -
2249	*Bacillus laterosporus*	**R2A** BLPM Cooper Cooper/Mn Cooper/Mg Cooper/Fe	+ + - - - +	+ - -
1229	*Paenibacillus sp.*	**EM** Xgen Cooper TSB	+ + + +	+ - + -

Continued Table 5.

MIK-SM number	Microorganisms	Media	Microbial Growth	Antimicrobial Activity
1230	*Paenibacillus sp.*	**EM**	+	+
		Xgen	+	+
		CooperMn	+	+
		TSB	+	+
		MSAM	+	-
		Bpm	+	-
		Landy	+	+
1473	*Paenibacillus burgundia*	**EM**	+	+
		Xgen	+	-
		Cooper/Mn	+	-
		TSB	+	-
		MSAM	-	-
		Bpm	+	+
		BHI	-	-
2158	*Streptomyces sp.*	OM	+	+
		Strept 1	-	
		Strept 2	-	
		Strept 3	+/-	-
		Cooper	+	+
2387	*Streptomyces nodusus*	**OM**	+	+
		Strept 1	+/-	+
		Strept 3	+	+
		Strept 4	+	-
		Strept 6	+	+
2163	*Streptomyces sp.*	**OM**	+	+
		Cooper	+	+
2375	*Micromonospora floridensis*	**OM**	+	+
		Mic 1	+	+
		Mic 10	+/-	+
		Mic 15	+	+
		PM-5	+	+
801	*Methylobacter sp.*	**R2A**	+	+
		Methylobacter	+	-
			-	
		CA	-	
		Mineral M.	+	-
		Colby	+	-
		Methyl Thio		
147	*Alcaligenes eutrophus*	**EM**	+	+
		CooperMn	+	-
		Alc 1	+/-	-
		Alc 2	+	-

Continued Table 5.

MIK-SM number	Microorganisms	Media	Microbial Growth	Antimicrobial Activity
195	*Alcaligenes sp.*	**EM**	+	+
		Alc 1	-	-
		Alc 2	+	-
		Cooper/Mn	+	-
307	*Pseudomonas fluorescens*	**R2A**	+	+
		KMB	+	-
		KMA	+	-
		M M	-	-
		YSE	+	-
		CooperZn/Fe	+	-
		F1 M	+	-
		F2 M	+	+
2271	*Burkholderia sp.*	**R2A**	+	+
		NBG	+	+
		LB	+	+
		PDB	+	+
707	*Klebsiella pneumonia*	**EM**	+	+
		M-9	-	-
		TSB	+	-
		BHI	+	-
		Cooper/Mn	+	-
743	*Klebsiella planticola*	**EM**	+	+
		M-9	-	-
		TSB	+	-
		BHI	+	-
		Cooper/Mn	+	-
931	*Arthrobacter agilis*	**R2A**	+	+
		Veg M	+	-
		EM	+	-
		Cooper/Mn	-	-
		M 53	+	-
		PDB	+	-
		Production	+	-
		GEN	+	-
		Vegetative	+	-
		F2	+	-
		TSYEM	+	-
		YSE	+	-
		Vegetative 2	+	-
		GYM	+	-
		YEG	+	+

Continued Table 5.

MIK-SM number	Microorganisms	Media	Microbial Growth	Antimicrobial Activity
991	*Arthrobacter sp.*	**EM** Vegetative Cooper/Mn M 53	+ + - +	+ - - -
1376	*Arthrobacter sp.*	**EM** Vegetative Cooper/Mn M 53	+ + - +	+ - - -
1387	*Arthrobacter sp.*	**EM** Vegetative Cooper/Mn M 53	+ + - +	+ - - -
1232	*Rhodococcus erythropolis*	**EM** M 53 CA M M GYM	+ + + - +	+ - - - -

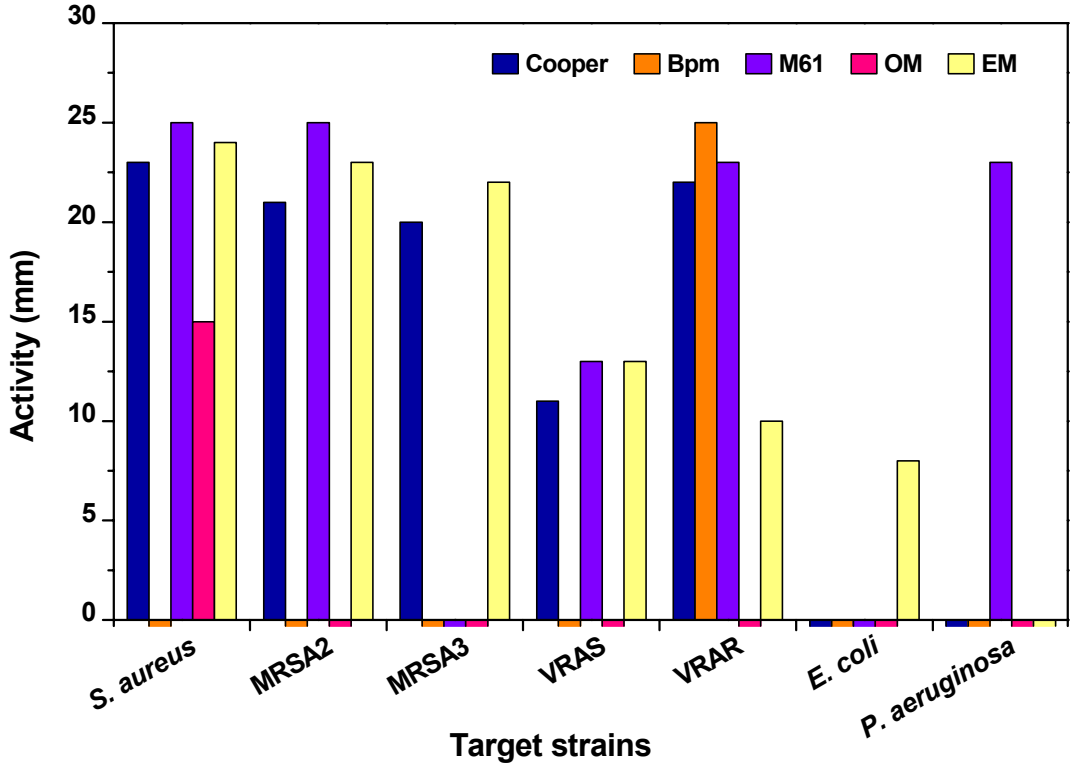
In bold are the media used for the production of the first extract and in which after storage of the strain no active secondary metabolite production was observed.
+ Optimal growth of the strain or antimicrobial activity in the corresponding media.
- Absence of growth of the strain or not antimicrobial activity in the corresponding media.
+/- Suboptimal growth of the strain in the corresponding media.

4.3. Analysis of secondary metabolite production in a *Bacillus laterosporus* strain

A standard procedure was established to optimise secondary metabolite production and subsequently, to identify the active compounds. This procedure, applied to several strains, will be illustrated with one of the most interesting strains, *B. laterosporus* MIK-SM 1242. The strain was isolated from Magadi Lake in Kenya and identified as *B. laterosporus* by 16S rRNA sequence analysis. The first extract, obtained by cultivating the strain in M61, showed interesting activities against multiresistant bacterial strains (methicillin resistant *S. aureus* and vancomycin resistant enterococci strains), fungi and yeast. After a second extract was prepared in the same medium and at same cultivation conditions, none of the

above mentioned activities were observed, indicating that the strain lost the capacity for producing corresponding secondary metabolites. For recovery this capacity eight different media were tested: Cooper, Xgen, *B. pumilus* medium, M61, OM, EM, OMLP and seed medium. The strain recovered the ability to produce antimicrobial secondary metabolites in 4 of the tested media (Figure 5). In some cases even antimicrobial activity not found in the first extract was induced by the new media. Most probably, the new media allowed other biosynthetic pathways thus production of other secondary metabolites not originally produced. A direct comparison of the effect of the cultivation media on the activity of whole MeOH extracts is a complicated task, due to their possibly different chemical composition. Finally, it was decided to use Cooper medium because when used for the cultivation of *B. laterosporus* strain, the obtained MeOH extract had an activity comparable to the one of the extracts obtained in other media and in addition presented the broadest antibacterial activity.

Figure 5. Antibacterial activity of the MeOH extracts produced by *Bacillus laterosporus* in different media.



M61: first extract produced, positive control.
MRSA: methicillin resistant *Staphylococcus aureus*
VRAS: vancomycin resistant-ampicillin sensitive enterococci
VRAR: vancomycin resistant-ampicillin resistant enterococci
Activity: antibacterial activity was tested in a diffusion agar assay (mm of inhibition).

Remarkably, in Cooper medium, an antibacterial peptide with activity against vancomycin resistant strains was produced by *B. laterosporus*. Further studies were performed in order to increase the production of this antibacterial peptide in order to allow its chemical characterization. For this purpose, Mg^{+2}, Zn^{+}, Mn^{+2}, Fe^{+} and DMSO were added to the Cooper media (Table 6).

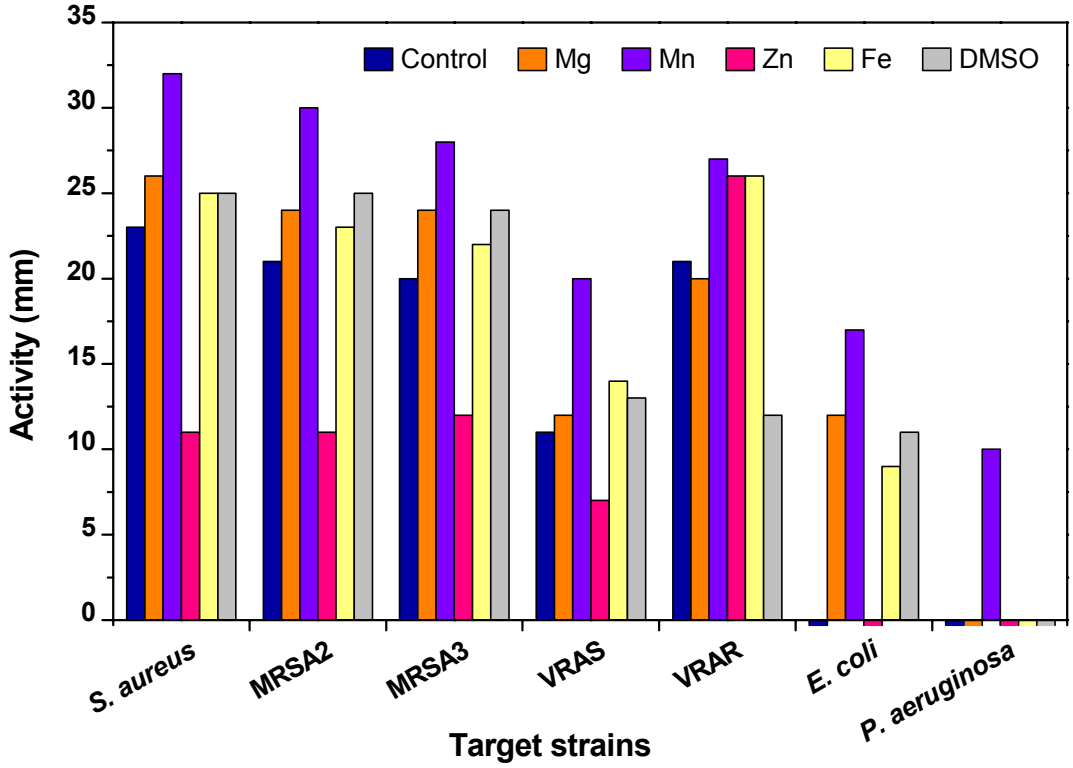
Table 6. Compounds used for promoting secondary metabolite production

Compound	**Product**	**Final Concentration***
Mn^{+2}	$MnSO_4$. $5H_2O$	0.004 %
Zn^{+}	$ZnCl_2$	0.01 %
Mg^{+2}	$MgSO_4$	0.05 %
Fe^{+}	$FeSO_4$	0.01 %
DMSO	DMSO	3 %

DMSO: Dimethyl sulphoxide
*Final concentration used in the media culture

Interestingly, the addition of Zn^{+} acted as a repressor of antimicrobial secondary metabolite production. Contrary, an increment of the activity was observed when Mn^{+2} was added to Cooper medium (Figure 6). Moreover, the addition of Mn^{+2} induced the production of secondary metabolites with activity against *P. aeruginosa,* not produced in the not supplemented Cooper medium. This activity was previously only observed in the MeOH extract obtained when the *B. laterosporus* strain was cultivated in M61 medium directly after the strain isolation. Finally, different concentrations of Mn^{+2} were tested (0.002, 0.004 and 0.008 %) but no significant differences were observed in the antibacterial activity of the obtained extracts. Further secondary metabolite productions were done by cultivating the strain in Cooper media with 0.004 % of Mn^{+2}.

Figure 6. Antibacterial activity of the MeOH extracts produced by *Bacillus laterosporus* in Cooper with compounds for promoting secondary metabolite production.



Control: Cooper medium without compounds for promoting secondary metabolite production
Concentrations: 0.05 % Mg^{+2}, 0.004 % Mn^{+2}, 0.01 % Zn^{+}, 0.01 % Fe^{+} and 3 % DMSO
MRSA: methicillin resistant *Staphylococcus aureus*
VRAS: vancomycin resistant-ampicillin sensitive enterococci
VRAR: vancomycin resistant-ampicillin resistant enterococci
Activity: antibacterial activity was tested in a diffusion agar assay (mm of inhibition).

Once the Mn^{+2} at 0.004 % was selected to induce secondary metabolite production in this strain, the composition of the Cooper/ medium was modified in order to study the influence of each component on the antibacterial properties of the extract obtained after cultivation of *B. laterosporus* strain (Table 7).

Table 7. Modifications in Cooper medium composition in order to increase the antibacterial activity.

	KH_2PO_4 (g/l)	Na_2HPO_4 (g/l)	NH_4NO_3 (g/l)	$MgSO_4$ (g/l)	$CaCl_2$ (g/l)	Glucose %	Mn^{+2} %
Cooper	4.1*	5.7	4	0.1	$8x10^{-4}$	3^{+}	0.004
C	1.5	5.7	4	0.1	$8x10^{-4}$	1	0.004
C1	1.5	5.7	4	0.1	$4x10^{-4}$	1	0.004
C2	1.5	5.7	4	0.1	$1.6x10^{-3}$	1	0.004
C3	1.5	5.7	4	0.05	$8x10^{-4}$	1	0.004
C4	1.5	5.7	4	0.2	$8x10^{-4}$	1	0.004
C5	1.5	5.7	2	0.1	$8x10^{-4}$	1	0.004
C6	1.5	5.7	6	0.1	$8x10^{-4}$	1	0.004
C7	1.5	3	4	0.1	$8x10^{-4}$	1	0.004
C8	1.5	8.5	4	0.1	$8x10^{-4}$	1	0.004

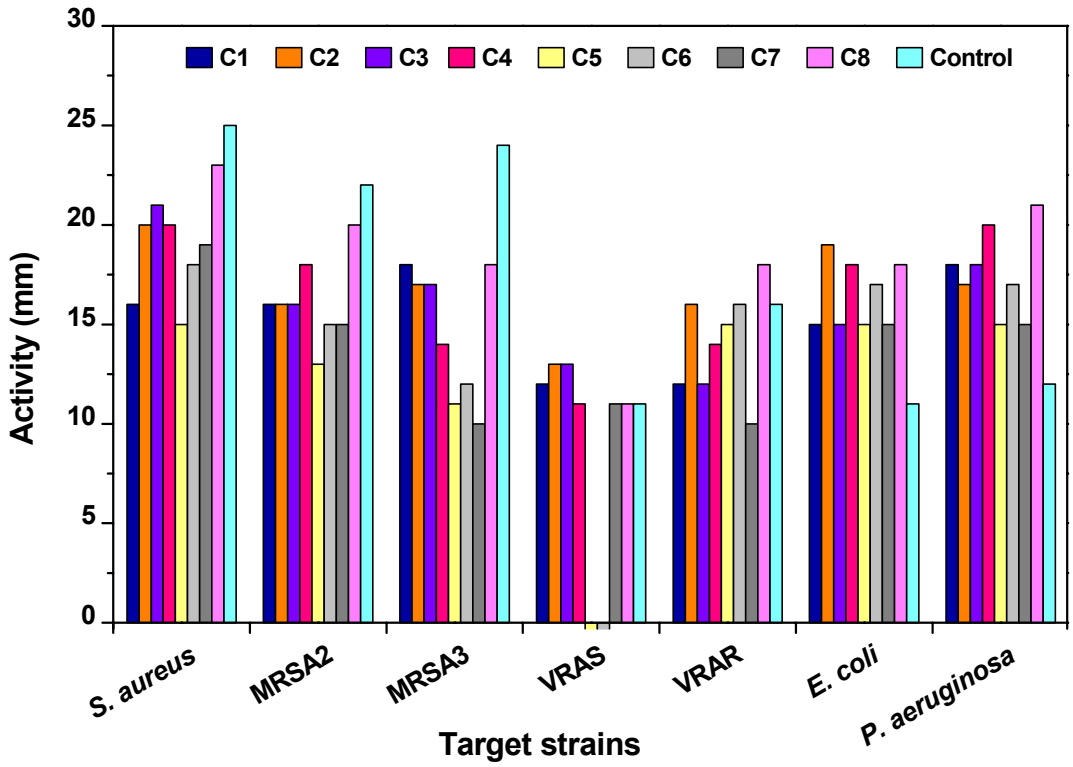
*4.1 g were the standard amount of KH_2PO_4 added to Cooper medium. In previous studies was observed that the same antibacterial activity was obtained with 1.5 g further reductions resulted in loss of activity.
$^{+}$3 % was the standard amount of glucose added to Cooper media. In previous studies was observed that the same antibacterial activity was obtained with 1 % further reductions resulted in loss of activity.
C: unmodified Cooper medium with 1 % glucose.
C1-C8: modified cooper medium with 1 % glucose.

The performed modifications affected the antibacterial activity of the produced MeOH extracts (Figure 7). However, a clear conclusion of the role of each component could not be withdrawn. Again, most probably, the metabolism of the *B. laterosporus* strain is affected by the different media composition and thus different secondary metabolites are

produced. However, by applying MeOH extracts on diffusion agar assays, the effect of media modifications on the overproduction of specific compounds found in the control medium or the production of a new compound can not be clarified due to the fact that the active compounds contained in the MeOH extracts act showing a synergistic effect.

Several of the extracts obtained by cultivating the *B. laterosporus* strain in modified Cooper medium were used for isolation and characterisation of active compounds. For instance, from the extract obtained with C8,which had the highest activity against *Pseudomonas aeruginosa*, Amicoumacin B, was isolated. This single compound was responsible for the activity against this strain. Additionally, from the extract obtained with medium C4 an antibacterial peptide with activity against multiresistant bacteria was detected. However, the amount of produced peptide was not enough to allow its identification.

Figure 7. Antibacterial activity of the MeOH extracts produced by *Bacillus laterosporus* in Cooper medium/Mn^{+2} with different composition.



Control: unmodified Cooper medium/ Mn^{+2}.
C1-C8: modifications to Cooper medium (Table 7)
MRSA: methicillin resistant *Staphylococcus aureus*
VRAS: vancomycin resistant-ampicillin sensitive enterococci
VRAR: vancomycin resistant-ampicillin resistant enterococci
Activity: antibacterial activity was tested in a diffusion agar assay (mm of inhibition).

4.4. Selection of microbial extracts with highest antimicrobial activities

From 81 selected producers, which extracts showed antibacterial activity in the first screening, 61 demonstrated good stability of secondary metabolite production. The antibacterial activity of these extracts against the different targets reference and clinical isolates was determined by both the microdilution and agar diffusion tests. In this form,

MeOH extracts with higher antimicrobial activity were selected for further studies. A highly active extract was considered when it showed more than 20 mm of inhibition of target strains in the agar diffusion test and showed activity until dilution 1/512 in the liquid test. A total of 29 MeOH extracts complied with the latter characteristics and were selected for further chemical characterisation. Specifically, 15 MeOH extracts were active against MRSA, 12 against VRE, 11 against gram negative strains and all against *S. aureus*,.

4.5. Isolation and chemical identification of the active compounds contained in the microbial extracts

Different methodologies were tested to find the most appropriate strategy to achieve rapid and efficiently the identification of the active substances contained in the different MeOH extracts produced during this study. For instance, TLC was used as a method for simultaneous evaluation of antimicrobial activity and subsequent isolation of the active substance. However, only few active compounds were isolated for identification using this methodology. Interestingly, TLC was also used to compare the pattern of activity of the producers that belong to the same genus. The strains producing the same active compounds (same TLC pattern of activities) were: two *Bacillus pumilus* (MIK-SM 977 and 988), two *Bacillus subtilis* (MIK-SM 1236 and 1238), three *Bacillus laterosporus* (MIK-SM 1242, 1243 and 1244) and two *Rhodococcus* sp. (MIK-SM 138 and 139) (Table 8). In addition, this procedure was useful for the determination of the appropriate solvent mixtures for the elution of the active compounds using liquid chromatography in silica columns. The latter mentioned was a tentative approach for the isolation and purification of the active substances. However, did not allow an optimum separation of the active compounds contained in the MeOH extract and further HPLC purifications were necessary. Therefore,

the use of chromatography in silica columns as a method for isolation of active compounds for chemical identification was discarded.

HPLC was the most successful strategy used for separation of the active compounds for identification purposes. Later, this was the routine approach used for the analysis of the active MeOH extracts. Indeed, a correlation between the biological activities and a zone in the chromatogram was directly performed in order to identify the corresponding active peak and determine its UV spectrum. A Further HPLC-UV-MS analysis allowed the determination of the molecular weight (MW) of the active compound. Finally, by means of a database comparison of obtained data (UV and MW) with those of known molecules, allows the prediction of the possible structure of the active substance. When not match with in the databases was found, further purification and NMR analysis were required in order to confirm the presence of a new molecule and elucidate its structure. Most of the compounds obtained in this study were characterised using this methodology (Table 8).

Table 8. Chemical identification of the antimicrobial compounds contained in MeOH extracts produced by 17 environmental isolates.

MIK-SM number	**Producer**	**Antimicrobial Activity**	**Recognised structures**
1	*Aeromicrobium erythreum*	*S. aureus*	Peptides
138=139	*Rhodococcus* sp.	*S. aureus*	Aromatic peptide
2580=2579	*Burkholderia* sp.	*S. aureus* MRSA 2 and 3 VRE *S. pombe* *B. cinerea*	Quinol derivatives: 2-Heptyl-4-quinolinone 2-Heptenyl-3-methyl-4-quinolinone 2-(2-nonenyl)-3-methyl-4-quinolinol Polyenes/ines
2271	*Bacillus* sp.	*S. aureus* MRSA 2 and 3 VRE *P. aeruginosa* *E. coli*	Amicoumacin B Amicoumacin C Bacillaene
969	*Bacillus thuringiensis*	*S. aureus* MRSA 2 and 3	Peptides
977=988	*Bacillus pumilus*	*S. aureus* MRSA 2 and 3 *P. digitatum* *R. oryzae*	Amicoumacin A Amicoumacin C Amicoumacin variant (mw=435) Unknown (mw=670)
2329	*Bacillus pumilus*	*S. aureus* MRSA 2 and 3	Amicoumacin A 4 polar variants of Amicoumcin A
1014	*Bacillus pumilus*	*S. aureus* MRSA 2 and 3 VRAR *P. aeruginosa* *E. coli* *F. fujikuroi* *R. oryzae*	Amicoumacin A Amicoumacin B Bacillaene Peptide: Surfactin or Pumiladicin B
2584=2587	*Bacillus subtilis*	*S. aureus* MRSA 2 and 3 VRE *P. aeruginosa* *E. coli* *B. cepacia* *S. pombe* *B. cinerea* *P.digitatum* *F. fujikuroi*	Difficidine Oxydifficidine Bacillaene

Continued Table 8.

MIK-SM number	**Producer**	**Antimicrobial Activity**	**Recognised structures**
234	*Bacillus subtilis*	*S. aureus* MRSA 2 and 3 VRAR *P. aeruginosa* *E. coli* *All tested fungi*	Bacillomycin
1236=1238	*Bacillus subtilis*	*S. aureus* MRSA 2 and 3 *F. fujikuroi*	Peptides with molecular mass above limit 1800.
1242=1243=1244	*Bacillus laterosporus*	*S. aureus* VRE *P. aeruginosa* *B. cepacia* *P. anomala* *M. pulcherrina*	Bogorol A Bogorol C Bacillaene Lipopeptides and peptides Amicoumacin B
2198a	*Bacillus ehimensis*	*S. aureus* MRSA 2 and 3 VRAR *P. aeruginosa*	Tetraene and polyene compounds.
2555	*Bacillus niacini*	*S. aureus* VRE *C. albicans* *C. glabrata* *C. anomala*	Moenomycin-B2 or Moenomycin-A12 Moenomycin A
2586	*Bacillus subtilis*	*S. aureus* MRSA 3 VRE *P. aeruginosa* *B. cepacia* *B. cinerea* *F. fujikuroi* *C. krusei* *C. tropicalis*	**Unknown macrolactin** Macrolactin A 7-*O*-Succinylmacrolactin A or F Bacillomycin D2 and D1 or D3 Oxydifficidin and Difficidin Surfactins Baccillomycin L1, L2 or L3 and L4 or L5
2162	*Streptomyces panayensis*	*P. digitatum* *P. anomala* *F. fujikuroi* *M. pulcherrina*	Pentaenes (3) Fungichromin (Lagosin) Elizabethin (Isolagosin) Filipin II Filipin III Filipin IV

Continued Table 8.

MIK-SM number	**Producer**	**Antimicrobial Activity**	**Recognised structures**
2198	*Streptomyces* sp.	*S. aureus* MRSA 2 and 3 VRAR *P. aeruginosa* *C. albicans* *C. glabrata* *C. anomala* *C. tropicalis* *P. anomala*	Actinomycin K1c Actinomycin X0d/X0B Pentaenes

MRSA: methicillin resistant *Staphylococcus aureus*
VRAR: vancomycin resistant-ampicillin resistant enterococci
VRE: vancomycin resitant enterococci

Only 17 from the 29 MeOH extracts with high antimicrobial activity were successfully chemically characterised. The remaining extracts were not efficiently characterised because either meagre concentration of active substance or an inefficient separation of the active compound, peak overlapping, with the standard chromatography method. No further separation was attempted with improved HPLC or other separation methods. In addition, most of the antibacterials identified as peptides were not further characterised because their chemical properties exceed the limit of the available equipment (molecular masses above 1800), active peptide did not show an UV spectrum or insufficient amount of the active peptide was obtained. In order to circumvent the latter problem, media modifications were attempted to increase production of active peptides but not significant improvements were obtained.

In conclusion, over 50 compounds were identified from the different MeOH extracts. However, only one compound was recognised as a possible novel molecule. Indeed, the UV data indicated that this compound was a macrolactin but its MW did not correlate with any of the already known macrolactins.

4.6. The *Bacillus subtilis* strain producer of a new antimicrobial macrolactin compound

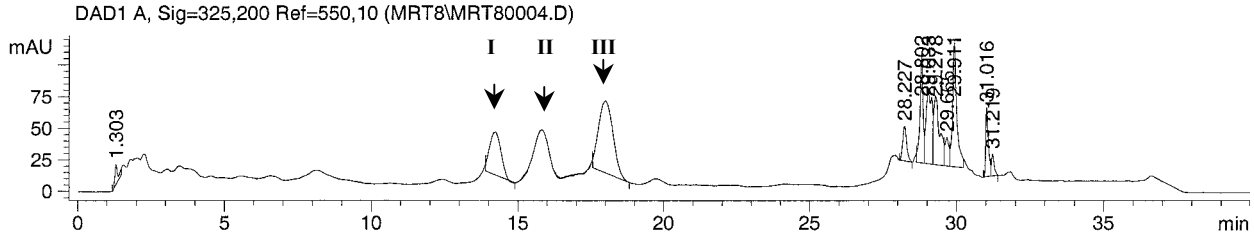
The strain *Bacillus subtilis* MIK-SM 2586 produced 3 macrolactins and several other antimicrobial compounds. One of the macrolactins was identified as a new macrolactin according to its UV and MS data (Table 8). The strain was isolated from a soil sample obtained from a farmyard at Takalar, South Sulawesi Province, Indonesia on April 2000. This strain was phenotypic and biochemically characterized with the API 20E and 50CH kits (Biomerieux, France). The 16S rRNA sequence homology searches in the FASTA system corroborated the identification of the strain. The colony was opaque, ondulating, roughly edged and milky white. The strain was a gram-positive endospore (d. 0.2 μm) forming rod (0.5-1 x 1-3 μm) and it was motile with flagella. Biochemically, the *B. subtilis* strain was positive for oxidase, ornithine, mannitol, VP, citrate, TDA and hydrolysis of starch tests. Additionally, it was negative for nitrate, lysine, H_2S production, glucose, xylose, ONPG, indole and ureasa tests. The producer *B. subtilis* strain was deposited in the DSMZ under the accession number DSM 16696.

4.7. Production, extraction and purification of macrolactin compounds produced by *Bacillus subtilis*

Cultures of the producer *B. subtilis* strain were grown in OM medium for 7 days at 30°C and a MeOH extract was prepared by the standard methodology previously described. The different compounds present in the MeOH extract were separated by RP-HPLC. A total of 60 fractions were obtained, which were tested for antimicrobial activity by the agar diffusion assay. The strain produced around 10 different antibacterial compounds, which –

according to UV and MS data − were identified as bacillomycins, difficidins, oxydifficidins, aromatic lipopeptides, macrolactin type compounds; and among the macrolactins, a not yet reported compound (Table 8). The retention times of macrolactins were 13.9, 15.4 and 17.6 min, for compounds **I**, **II** and **III**, respectively. Compound **II** was the uncharacterised macrolactin (Figure 8). Efforts were concentrated in the production of enough amount of the three macrolactins required for the structural analysis and determination. A MeOH extract was produced from 4 L culture medium and the macrolactins separated by preparative HPLC. The fractions containing the three macrolactins were further purified by LH-20 chromatography. The yields from 4 L culture medium were 4-6 mg, 5-7 mg and 6-8 mg of compounds **I**, **II** and **III**, respectively.

Figure 8. HPLC chromatogram of a MeOH extract containing the macrolactin compounds.



4.8. Structural determination of the three macrolactin compounds produced by *Bacillus subtilis*

The molecular ion *m/z* 402 and the UV absorptions at 227 and 261 nm enabled the identification of compound **I** as macrolactin A or its 10*E*-isomer, macrolactin I. The latter was ruled out by its optical rotation of $[\alpha]^{22}_{D}$ = −138 compared to about −10 found for

macrolactin A (**I**). A direct comparison of the NMR data of **I** was difficult, because only ^{1}H-NMR data in benzene-d_6 and ^{13}C-NMR data in pyridine-d_5 are available in the literature. The ^{1}H- and ^{13}C-NMR data of the well resolved spectra of macrolactin A in MeOH-d_4 are given in Table 9. The signals were identified by ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-HMBQ spectra.

Chemical properties of Macrolactin A (I) produced by *B. subtilis*: $C_{24}H_{35}O_5$, M = 402.53, UV (MeOH): λ_{max} (lg ε) = 227 (4.537), 261 (4.146) [Lit.: 227 (4.691), 261 (4.272)]. $[\alpha]^{22}_{D}$ = − 10.7 (*c* = 0.68 in MeOH) [Lit.: − 9.6 (*c* = 1.86)]. MS: (−)-ESI (TOF): *m/z* (%) = 401.2 (38) $[M-H]^-$, 437.2 (100) $[M+Cl]^-$, 803.4 (63) $[2M-H]^-$; (−)-DCI (isobutane): *m/z* (%) = 402 (100); (+)-DCI (isobutane): *m/z* (%) = 349 (56) $[M+H-3\ H_2O]^+$, 367 (100)) $[M+H-2\ H_2O]^+$, 385 (75)) $[M+H-H_2O]^+$; EI (200 °C): *m/z* (%) = 255 (100), 273 (72), 348 (18), 366 (68), 384 (60), 400 (5.9), 402 (2.5). NMR data are given in Table 9.

Compound **II** was identified as a macrolactin A type compound from its identical UV spectrum. Mass spectrometry indicated the molecular mass of 488, which is 86 a.m.u higher than the mass observed for **I**. Corresponding to the elimination of one H_2O from **I**, compound **II** showed the loss of malonic acid by a fragment-ion at *m/z* 383 in the (−)-ESI spectrum. The NMR data of **II** in MeOH-d_6 were nearly identical to **I**. However, compared to **I** the 7-H signal was shifted about 1.2 ppm downfield as consequence of the acylation of 7-O. The residue at 7-O was identified from the ^{1}H- and ^{13}C-NMR spectra by comparison with **I**. Because only one carboxy group was directly visible in the NMR spectra of **II** in MeOH-d_4, a set of NMR spectra in dichloromethane-d_2 clearly indicated the malonyl

residue by additional carboxy ^{13}C signals at 166.33 and 169.15 ppm and a methylene ^{13}C signal at 42.17 ppm, which was correlated to two ^{1}H doublet signals at 3.51 and 3.40 ppm (J = 15.5 Hz). NMR data and chemical structure of the new compound are given in Table 10, Figure 9 and Figure 10.

Table 9. NMR data of macrolactin A (**I**) in MeOH-d_4 (^{1}H- and ^{13}C).

H	δ_H	m	J	C	δ_C	m
1	-	-	-	1	168.02	s
2	5.58	d	11.33	2	118.00	d
3	6.67	t	11.71	3	144.96	d
4	7.26	dddd	15.1, 11.4, 2, 1	4	130.26	d
5	6.20	m	-	5	142.16	d
6	2.45	m	-	6	42.84	t
7	4.29	ddt	4.9, 1.2, 6.8	7	72.33	d
8	5.79	dd	15.1, 6.0	8	137.55	d
9	6.61	ddt	15.2, 11.0, 1.1	9	125.96	d
10	6.15	t	11.14	10	131.39	d
11	5.58	ddd	10.5, 8.6, 8.2	11	128.39	d
12a	2.53	dddd	13.5, 8.2, 7.4, 0.8	12	36.50	t
12b	2.36	dddd	13.5, 7.7, 4.9, 1.1			
13	3.89	ddt	5.3, 5.1, 6.9	13	69.24	d
14	1.65	m	-	14	43.92	t
15	4.34	dt	6.3, 6.3	15	69.83	d
16	5.60	dd	15.1, 6.5	16	135.23	d
17	6.21	dd	15.5, 10.9	17	131.21	d
18	6.09	dd	14.9, 10.4	18	131.72	d
19	5.69	ddd	14.7, 7.2, 6.8	19	135.13	d
20a	2.23	ddt	14.2, 7.1, 6.8	20	32.98	t
20b	2.14	ddt	14.2, 6.4, 7.1			
21	1.54	m	-	21	25.65	T
22a	1.68	m	-	22	36.01	T
22b	1.61	m	-			
23	5.05	ddq	7.3, 4.5, 6.2	23	72.21	D
24	1.29	d	6.04	24	20.11	Q

^{1}H at 600 MHz; ^{13}C at 150 MHz; multiplicity of carbon signals from DEPT and HMQC spectra.

Chemical properties of 7-*O*-malonyl macrolactin A (7-O-m) (II) produced by *B. subtilis*: $C_{27}H_{36}O_8$, M = 488.57, UV (MeOH) (Figure 9): λ_{max} (lg ε) = 227 (4.397), 230 (sh), 260 (4.006). $[\alpha]^{22}_D$ = − 6.2 (*c* = 0.63 in MeOH). MS: (−)-ESI (TOF):): *m/z* (%) = 487.2 (100) $[M-H]^-$, 443.2 (44) $[M-H-CO_2]^-$, 383.2 (27) [M−H−malonic acid]$^-$; (Table 10).

Figure 9. UV spectrum of 7-*O*-malonyl macrolactin A (**II**) in MeOH

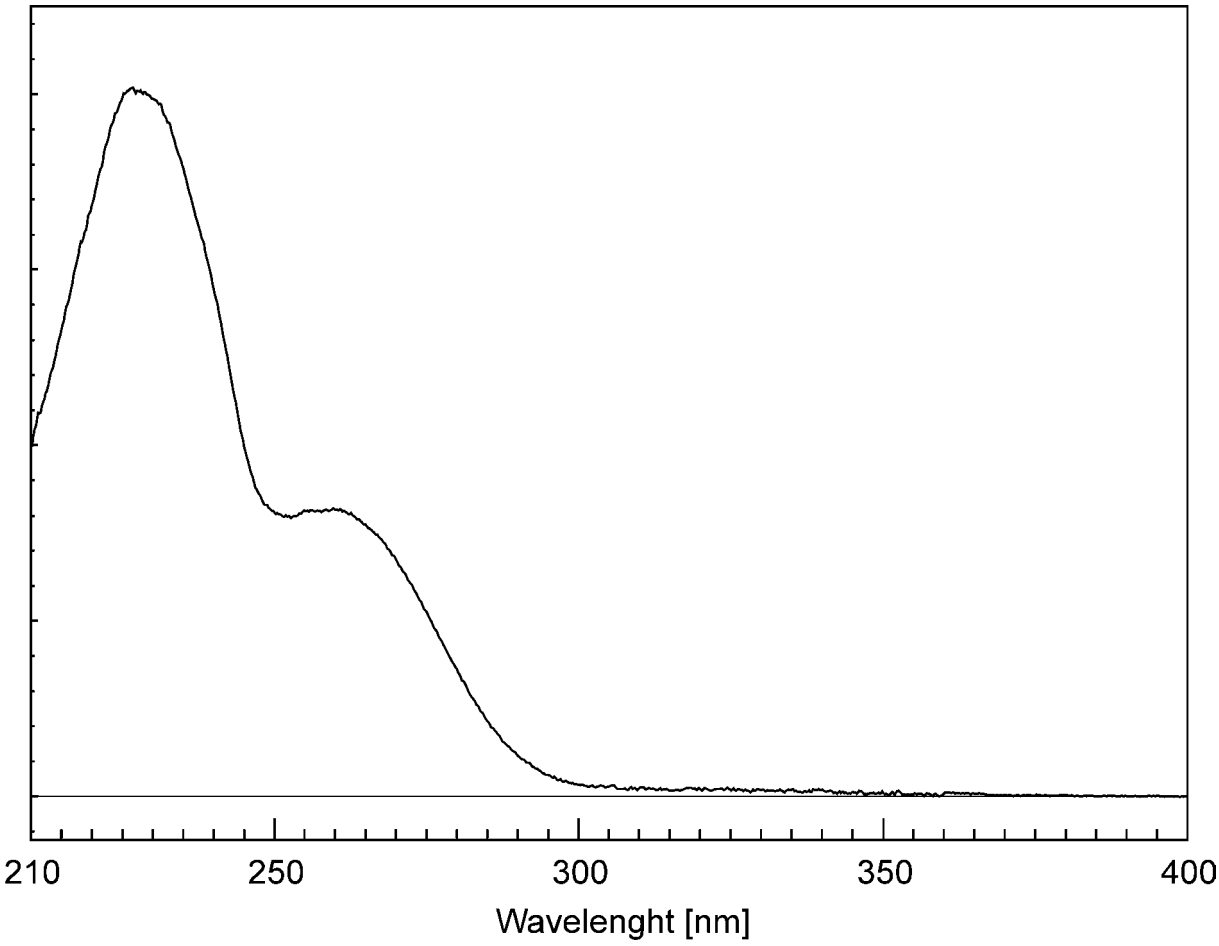
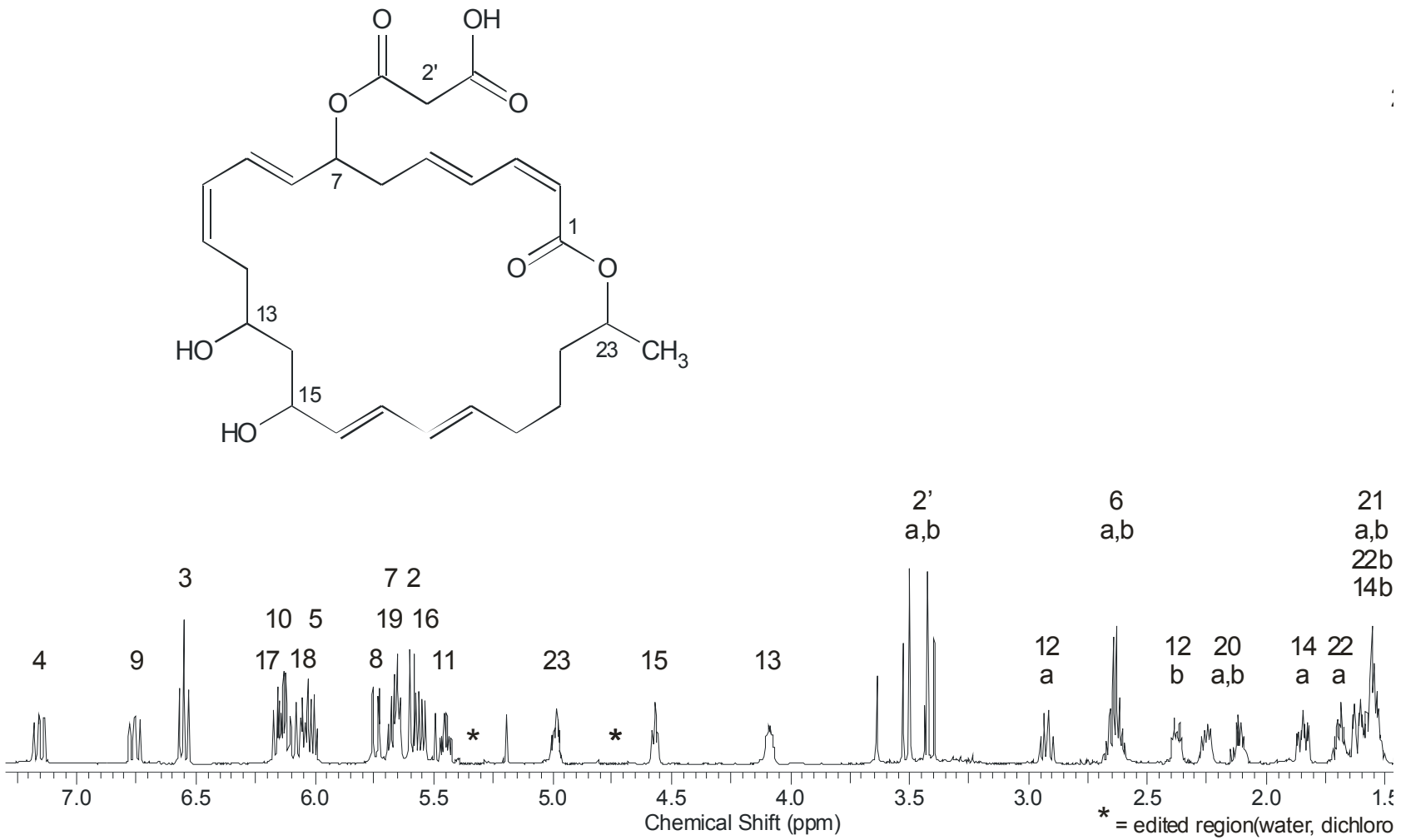


Table 10. NMR-Data of 7-*O*-malonyl macrolactin A (**II**) in MeOH-d_4 (^{1}H- and ^{13}C).

H	δ_H	m	*J* [Hz]	C	δ_C	m
1	-	-	-	1	167.94	s
2	5.59	d	11.7	2	118.52	d
3	6.67	t	11.3	3	144.50	d
4	7.25	dd	14.7, 11.7	4	130.79	d
5	6.15	dt	15.4, 7.2	5	140.51	d
6	2.60	m	5.3	6	40.13	t
7	5.50	ddd	6.0, 6.0, 6.0	7	74.72	d
8	5.75	dd	15.3, 5.5	8	132.06	d
9	6.71	dd	15.1, 11.3	9	128.09	d
10	6.13	t	10.2	10	130.91	d
11	5.63	dt	10.6, 8.4	11	129.78	d
12a	2.63	m	-	12	36.39	t
12b	2.33	ddd	13.0, 7.2, 5.5			
13	3.84	ddd	10.6, 6.0, 5.7	13	69.51	D
14	1.66	m	-	14	43.84	T
15	4.39	dt	6.3, 6.3	15	69.77	D
16	5.60	dd	15.1, 6.4	16	135.32	D
17	6.21	dd	15.1, 10.6	17	131.27	D
18	6.10	dd	15.1, 10.6	18	131.78	D
19	5.69	ddd	14.9, 7.0, 6.8	19	135.10	D
20a	2.23	td	14.0, 6.8	20	33.03	T
20b	2.15	td	14.4, 7.2			
21	1.54	m	-	21	25.81	T
22a	1.70	m	-	22	36.08	T
22b	1.62	m	-			
23	5.05	ddq	4.5, 7.1, 6.1	23	72.37	D
24	1.30	d	6.0	24	20.14	Q
1’	-	-	-	1’	169.64	S
2’	2.90	m	- (br)	2’	44.74	(a)
3’	-	-	-	3’	n.o.	

^{1}H at 600 MHz; ^{13}C at 150 MHz;
(a) from HMQC NMR spectrum.
n.o.= not observed due to signal broadening

Figure 10. H-NMR spectrum and chemical structure of 7-*O*-malonyl macrolactin A (**II**) in dichloromethane-d_2 (600 MHz)

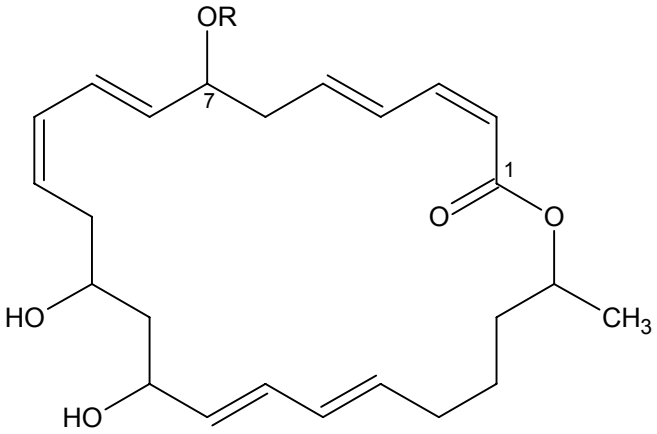


Compound **III** was identified as 7-*O*-succinyl macrolactin A. The ^{1}H NMR data of **III** in $CDCl_3$ were found to be identical to those in literature [62].

Chemical properties of 7-*O*-succinyl macrolactin A (7-O-s) (III) produced by *B. subtilis*: $C_{28}H_{38}O_8$, M = 502.60: UV (MeOH): λ_{max} (lg ε) = 227 (4.596), 259 (4.192) [Lit.: 229 (4.57), 261 (4.18)]. $[\alpha]^{22}_{D}$ = − 19.9 (*c* = 0.7 in MeOH) [Lit.: − 9.6 (*c* = 0.18 in MeOH)]. MS: (−)-ESI (TOF): *m/z* (%) = 501.28 (100) $[M-H]^-$, 117.02 (12) [succinic acid−H]$^-$; (−)-DCI (isobutane): *m/z* (%) = 502.7 (100), 484 (44), 402 (18), 384 (68), 366 (26), 117 (20).

In Figure 11 the chemical structures of the macrolactins produced by *B. subtilis* are compared. The difference between the three compounds is in the residue in C7.

Figure 11. Chemical structure of the macrolactins purified from the MeOH extract produced by *Bacillus subtilis*.



Macrolactin A (**I**) R = H

7-*O*- malonyl macrolactin A (**II**) R = CO-CH_2-COO

7-*O*- succinyl macrolactin A (**III**) R = CO-CH_2-CH_2-COOH

4.9. Optimisation of macrolactin production by *Bacillus subtilis* strain

The effect of some cultivations parameters (e.g. pH, media composition) on the macrolactin production by *B. subtilis* was studied. Activities of the obtained MeOH extracts were tested against several multiresistant strains by diffusion agar test. In addition, it was proved that *Staphylococcus aureus* was exclusively sensitive to the macrolactin fractions of the MeOH extracts. In this form, the inhibition zone obtained with the whole extract could be correlated specifically to the macrolactin activity. Moreover, calibration curves were done with purified macrolactins for quantitative purposes. Finally, chromatograms of each extract were always compared to ensure that the overall pattern did not vary considerably with the new conditions and that main changes were observed in the peaks corresponding to the macrolactins. Important is to mention that traces of compounds with chemical characteristics similar to those of the macrolactin, similar retention times, were often found.

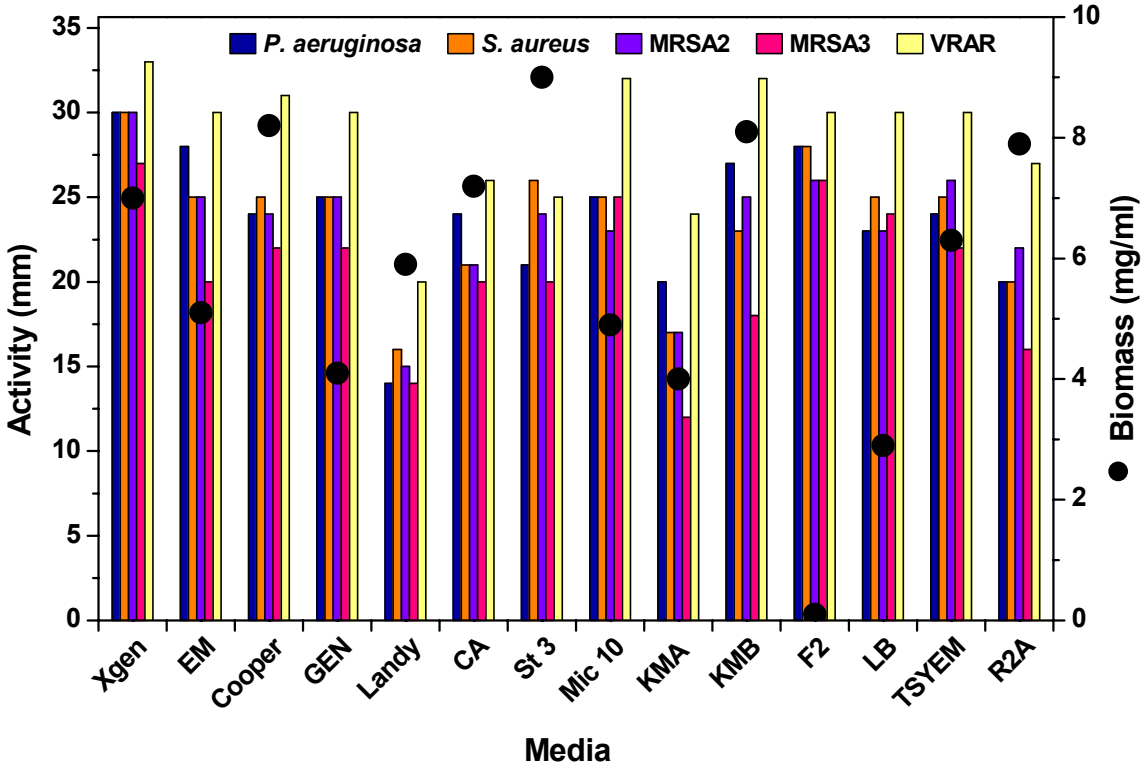
4.9.1. Media selection for macrolactin production

A total of 20 different media were tested in order to find the conditions appropriate for macrolactin production. The tested media were selected containing different carbon and nitrogen sources as well as different trace elements, vitamins and amino acids.

The *B. subtilis* strain grew in all tested media, but St-1, SMM, M61 and seed medium. Interestingly, in Bpm the strain was able to grow, however, the obtained extract was not active against the tested strains. In contrast, all other tested media which were able to support *B. subtilis* growth allowed also production of antibacterial secondary metabolites with activities against the bacterial target strains and specially against vancomycin resistant-ampicillin resistant enterococci (VRAR) (Figure 12). Of particular interest was

the high antibacterial activity observed in the MeOH extracts produced in F2 and OM media which contrasted with the scarce biomass obtained. This independence of the biological activity and the produced biomass was frequently observed during the whole study.

Figure 12. Antibacterial activity of the MeOH extracts and biomass produced by *Bacillus subtilis* strain in different media.



MRSA: methicillin resistant *Staphylococcus aureus*
VRAR: vancomycin resistant-ampicillin resistant enterococci
Activity: antibacterial activity was tested in an agar diffusion assay (mm of inhibition).
In the graphic are only included the media in which activity was detected.

The macrolactin concentration of the produced extracts was deduced from the antimicrobial activity against *S. aureus*. Highest macrolactin production was observed in OM, F2 and Xgen media (Table 11). This result was confirmed by comparing the HPLC chromatograms of the extracts. Indeed, similar chromatogram patterns were obtained from the different media and only differences were found in the peaks corresponding to the macrolactins. For further studies OM medium was selected since traces of several compounds with chemical characteristics similar to the macrolactins, which interfered with the purification process, were kept at minimum.

Table 11. Quantification of macrolactins production by *Bacillus subtilis* strain grown in different media.

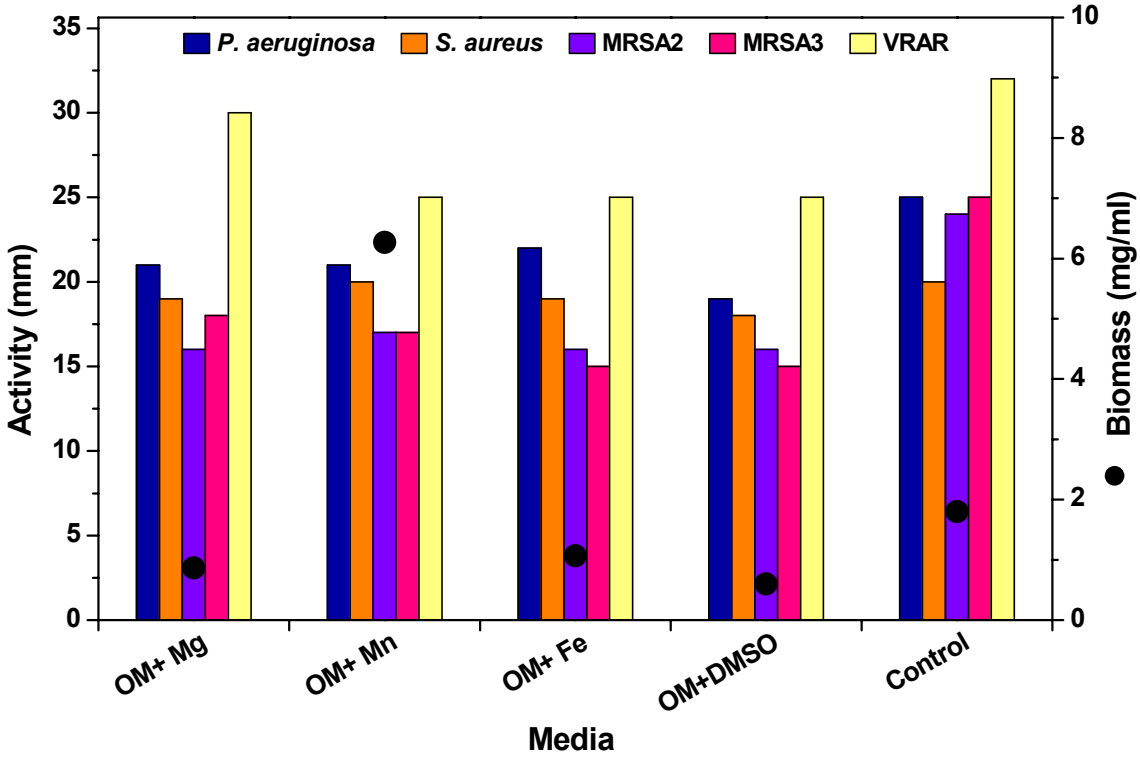
Media	**Macrolactins µg /µl**
Xgen	3.7
EM	3.1
Cooper	3.1
GEN	3.1
Landy	1.7
CA	2.4
St-3	3.1
Mic-10	3.1
KMA	1.7
KMB	2.8
F2	3.7
LB	3.1
TSYEM	3.1
R2A	2.2
OM	3.7

4.9.2. Addition of compounds for promoting macrolactin production

Optimisation of macrolactin production by *Bacillus subtilis* in OM medium was attempted by modifying the media composition with the addition of the compounds for promoting secondary metabolite production reported in the literature [12, 63, 64]. However, these

modifications did not have a positive effect on the activity of the obtained MeOH extracts (figure 13). In fact, in some cases media modifications negatively affected the antibacterial activity of the produced MeOH extracts. From the antibacterial activity against *S. aureus*, macrolactin concentration was calculated as 3.0±0.2 μg/μl in all tested media. Interestingly, some effects of the compounds tested for promoting secondary metabolite production were observed on the biomass. When Zn^{2+} was added to the medium, no growth of the strain was detected. In contrast, in the presence of Mn^{2+}, the production of biomass increased significantly in comparison to the other media. For further studies OM medium without additives was retained as the production medium.

Figure 13. Antibacterial activity of extracts and biomass produced by *Bacillus subtilis* in OM medium with addition of compounds for promoting secondary metabolite production. Control: OM medium without compounds for promoting secondary metabolite production



Concentrations: 0.05 % Mg^{+2}, 0.004 % Mn^{+2}, 0.01 % Zn^{+}, 0.01 % Fe^{+} and 3 %DMSO
MRSA: methicillin resistant *Staphylococcus aureus*
VRAR: vancomycin resistant-ampicillin resistant enterococci
Activity: antibacterial activity was tested in an agar diffusion assay (mm of inhibition)
Addition of Zn^{+} resulted in not growth

4.9.3. Influence of the OM medium composition in the macrolactin production

The influence of the different components of the OM medium on biomass, secondary metabolite and macrolactin production was evaluated. Modifications were made by either removing one component, increasing 5 fold concentration of one component (retaining all other components) or increasing the concentration of all the components (Table 12).

Table 12. Modifications to the composition of OM medium.

	OM Medium							
	Starch (g/l)	Glucose (g/l)	Peptone (g/l)	Yeast extract (g/l)	Solutions (ml/l):			
					A	B	C	D
C	1	1	1	1.5	10	10	1	1
OM1	0	1	1	1.5	10	10	1	1
OM2	5	1	1	1.5	10	10	1	1
OM3	1	0	1	1.5	10	10	1	1
OM4	1	5	1	1.5	10	10	1	1
OM5	1	1	0	1.5	10	10	1	1
OM6	1	1	5	1.5	10	10	1	1
OM7	1	1	1	0	10	10	1	1
OM8	1	1	1	7.5	10	10	1	1
OM9	5	5	5	7.5	10	10	1	1
OM10	1	1	1	1.5	10	0	1	1
OM11	1	1	1	1.5	10	30	1	1
OM12	1	1	1	1.5	10	10	0	1
OM13	1	1	1	1.5	10	10	3	1
OM14	1	1	1	1.5	10	10	1	0
OM15	1	1	1	1.5	10	10	1	3

Continued Table 12.

	OM Medium							
	Starch (g/l)	Glucose (g/l)	Peptone (g/l)	Yeast extract (g/l)	Solutions (ml/l):			
					A	B	C	D
OM16	1	1	1	1.5	10	0	0	0
OM17	1	1	1	1.5	10	30	3	3

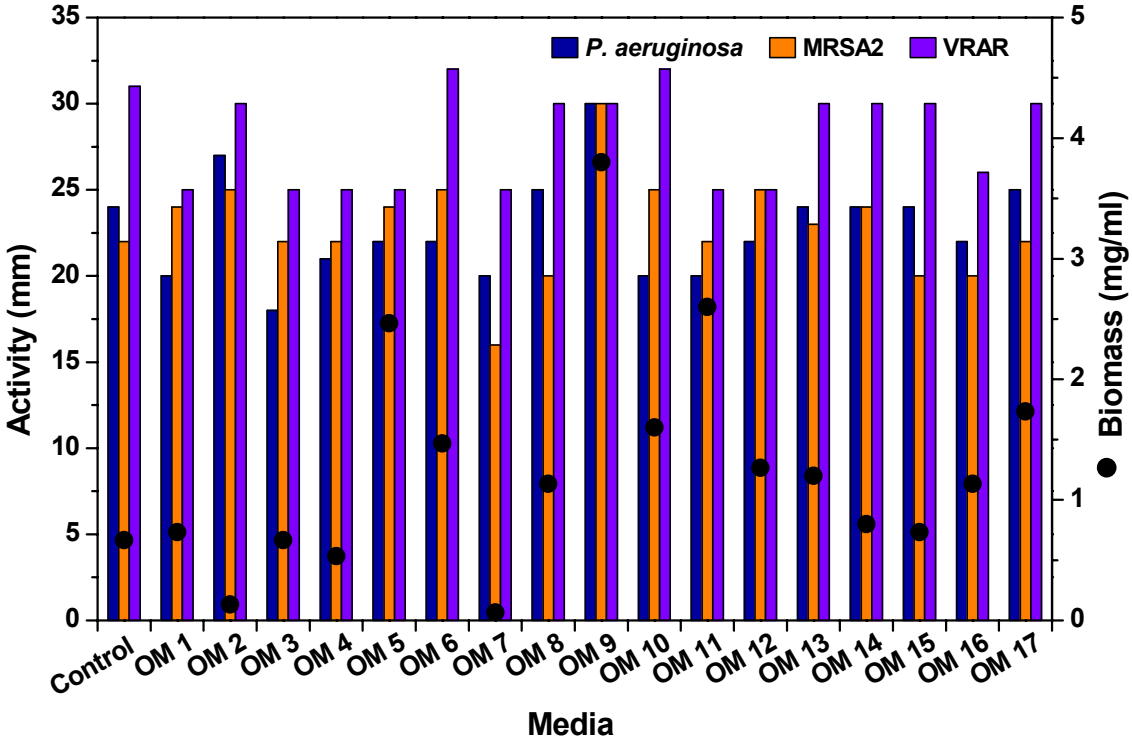
C: OM medium standard composition
OM1-17: OM medium with modified composition
Solutions A, B, C and D: see appendix media
Solution A buffer without variations (media pH)

The biomass production was highly influenced by the cultivation media. Maximum biomass production was obtained in OM9 (Figure 14), where all the components, except the solutions, were increased.

The effect of the medium composition in the biological activity of the macrolactins was evaluated determining: a) the antibacterial activity of the obtained extracts against target strains using agar diffusion tests (Figure 14) and b) quantification of the amount of macrolactin produced using calibration curves (Figure 15). Despite the great differences in media composition, all obtained MeOH extracts presented antibacterial activity. Similar to the biomass production, the antibacterial activity varied depending on the proportions of the reagents. Highest activity against the target strains was produced in OM9 medium (Figure 14). Concerning the macrolactin production, highest amounts were produced in OM2, OM9 and OM17 media (Figure 15). Interestingly, in OM2 medium the biomass production was low, however, high antibacterial activity and macrolactin production were obtained. Macrolactin production was the same in OM2, OM9 and OM17, but the extracts

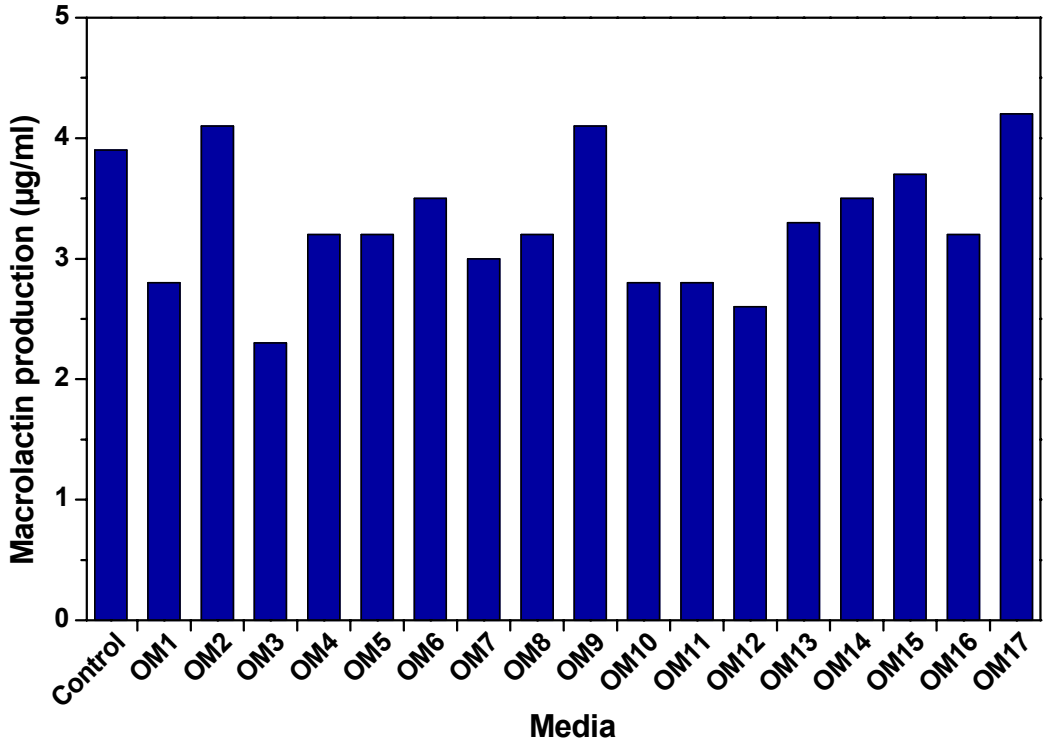
from cultures of OM2 and OM17 gave products with retention times similar to one of the macrolactins, which would complicate the purification process of the macrolactin, and thus these media were discarded.

Figure 14. Antibacterial activity of the MeOH extracts containing macrolactins and biomass produced by *Bacillus subtilis* in OM medium with different composition



Control: OM medium with unmodified composition
OM 1-17: medium with modified composition described in Table 12
MRSA: methicillin resistant *Staphylococcus aureus*
VRAR: vancomycin resistant-ampicillin resistant enterococci
Activity: antibacterial activity was tested in an agar diffusion assay (mm of inhibition).
Behaviour of the activities against MRSA3, *S. aureus* and *P. aeruginosa* were similar to those against MRSA2

Figure 15. Quantification of Macrolactin production by the *Bacillus subtilis* grown in OM medium with different composition

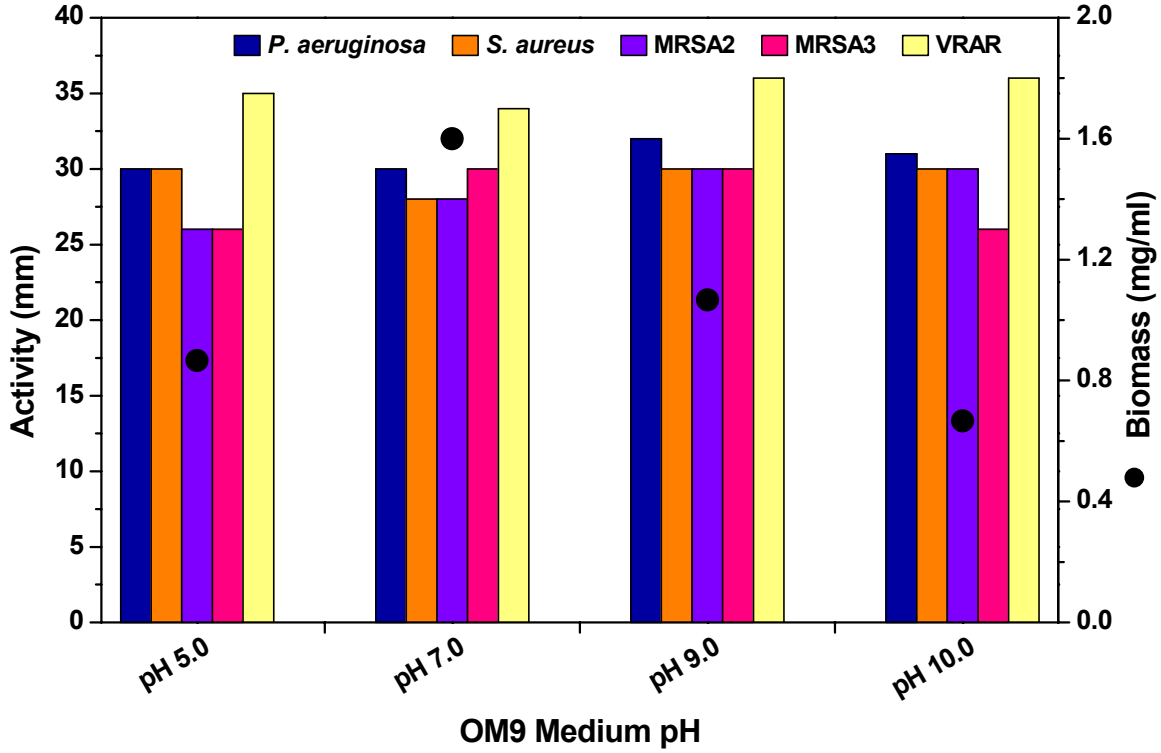


Control: OM medium with unmodified composition
OM 1-17: medium with modified composition described in Table 12

4.9.4. Effect of medium pH in macrolactin production by *Bacillus subtilis*

B. subtilis strain cultivated in OM9 medium, was able to grow in a wide pH range, which is one of the *Bacillus* genus characteristics. Optimum biomass production was obtained at pH 7.0 and any deviation from this value considerably affected cell growth. Interestingly, the antibacterial activity against the target strains and also the macrolactin production were not correlated with the amount of produced biomass. Similar antibacterial activity (Figure 16) as well as macrolactin production (6.8 µg/µl) was obtained at the different pH conditions.

Figure 16. Antibacterial activity of the MeOH extracts and biomass produced by *Bacillus subtilis* in OM9 medium at different pH conditions.



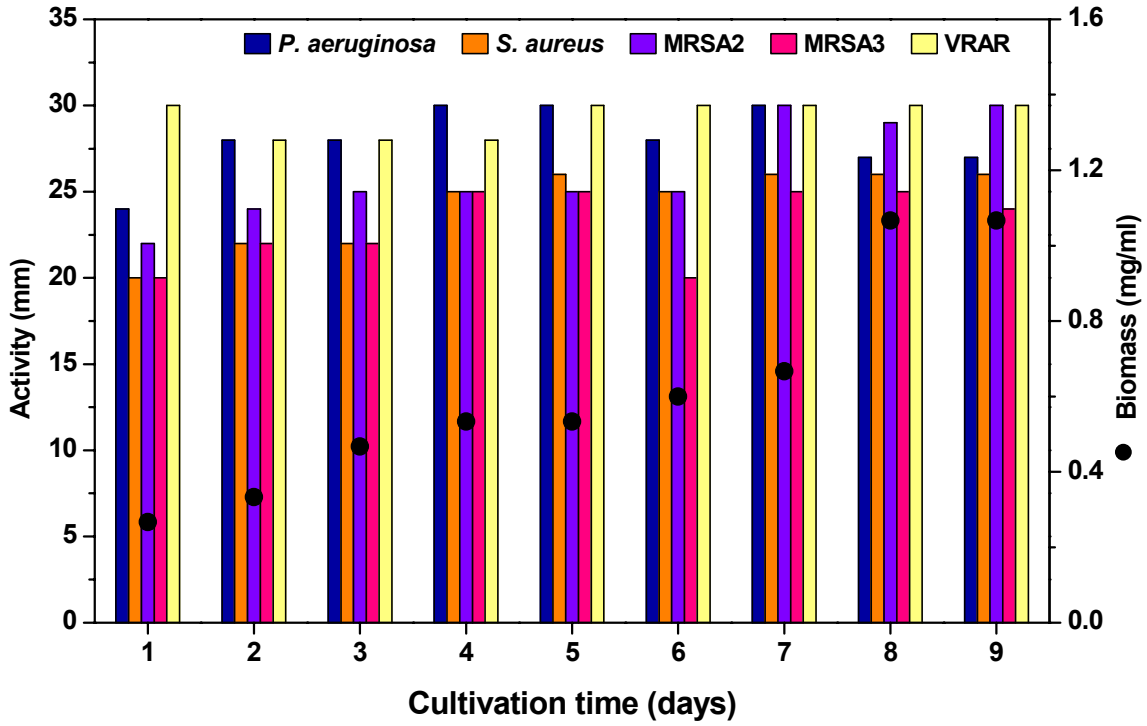
MRSA: methicillin resistant *Staphylococcus aureus*
VRAR: vancomycin resistant-ampicillin resistant enterococci
Activity: antibacterial activity was tested in an agar diffusion assay (mm of inhibition).

4.9.5. Macrolactin production by the *Bacillus subtilis* strain during 9 days of growth

The biomass production of *B. subtilis* and antibacterial activity of the obtained MeOH extracts were monitored during 9 days of incubation. Cell growth and antibacterial compounds production reached a maximum between 8-9 and 7-8 days respectively. In

contrast, the macrolactin production reached the maximum after five days of cultivation (Table 13). Remarkably, most of the secondary metabolite production was achieved during the first day of cultivation (Figure 17). No degradation of macrolactins and other antibacterial compounds over the 9 production days was observed, due to the protection conferred by the adhesion of the metabolites to the amberlite resin XAD-16.

Figure 17. Antibacterial activity of the MeOH extracts and biomass produced by *Bacillus subtilis* in OM9 medium during 9 days.



MRSA: methicillin resistant *Staphylococcus aureus*
VRAR: vancomycin resistant-ampicillin resistant enterococci
Activity: antibacterial activity was tested in an agar diffusion assay (mm of inhibition).

Table 13. Quantification of macrolactins production by the *Bacillus subtilis* grown in OM9 medium during 9 days

Time (days)	Macrolactins µg /µl
1	3.2
2	3.5
3	3.5
4	4.1
5	4.3
6	4.1
7	4.3
8	4.3
9	4.3

In summary, *B. subtilis* cultivation conditions for macrolactins production were found to be optimal in OM9 medium at pH 7.0. In this conditions maximum macrolactin concentration was obtained after 5 days of cultivation. The latter was the standard procedure for producing macrolactin during the rest of the present study.

4.10. Antimicrobial activities of the macrolactins compounds

The three macrolactins produced by the *B. subtilis* strain were purified by several chemical extractions followed by preparative RP-HPLC and LH-20 chromatography (see materials and methods). The biological activity of each macrolactin was investigated against a broad spectrum of microorganisms including reference strains and clinical isolates, by means of agar diffusion assay. The new macrolactin (compound **II** = 7-*O*-malonyl macrolactin A) was the most active macrolactin compound produced by the *B. subtilis* strain. In the agar diffusion assay, a large zone of inhibition of bacterial growth was observed around the disc (Table 14). However, growth inhibition was not complete and little colonies could be detected inside the zone of compound activity. The higher inhibition values obtained with

the MeOH extract were consequence of the additive effect of the three macrolactins and the other secondary metabolites with antibacterial activity produced by the strain (Table 14). 7-*O*-malonyl macrolactin A (7-O-m) showed antimicrobial activity against a wide number of strains. The obtained antimicrobial activity was comparable or superior to that of erythromycin (Table 14). Remarkably, 7-O-m was able to inhibit the growth of methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE) clinical strains.

Table 14. In vitro activities of the macrolactins produced by *Bacillus subtilis* in comparison with the MeOH extract and commercial antibiotics.

Strain	Inhibition zone size (mm)						
	MeOH extract [a]	**I** macrolactin A[b]	**II** 7-*O*-m[b]	**III** 7-*O*-s[b]	ERY[c]	VAN[d]	AMP[e]
S. aureus DSM 1104	28	18	26	18	30	20	30
S. aureus 32	32	25	30	22	40	27	41
MRSA2	24	27	40	37	0	24	0
MRSA3	28	35	41	38	0	24	0
E. faecalis	18	0	25	12	25	24	32
E. faecalis (VRAS)	15	0	15	0	0	0	35
E. faecium (VRAR)	40	0	15	20	25	0	0
B. cepacia SCV	40	0	15	0	0	ND	0

[a]10 µl/disc , [b]50 µg/disc, [c]78 µg/disc, [d]30 µg/disc and [e]10 µg/disc
In table are shown only the target reference and clinical strains against which high activity was found
7-O-m: 7-*O*-malonyl macrolactin A
7-O-s: 7-*O*-succinyl macrolactin A
MRSA: Methicillin-resistant *Staphylococcus aureus*
VRAS: Vancomycin-resistant Ampicillin-sensitive
VRAR: Vancomycin-resistant Ampicillin-resistant
SCV: Small colony variant
ND: not determined

4.11. Minimal Inhibitory Concentrations of the 7-*O*-malonyl macrolactin A

Minimal inhibitory concentrations (MIC) of 7-O-m against the target strains were determined by the standard twofold serial MH broth microdilution method described by the National Committee for Clinical Laboratory Standards (NCCLS) [54]. For many strains the MIC was not reached within the used concentration range (Table 15). However, very interestingly, the growth of some of these strains was affected already at relative low macrolactin concentration, to an extent that almost no turbidity was detected. For instance, the MIC of the 7-O-m for staphylococci were higher than 128 μg/ml, but a strong inhibition of the bacterial growth was observed already at concentrations of 1 and 4 μg/ml for the reference and for the MRSA strains respectively.

A similar effect was observed against all the enterococci strains. Indeed the MIC stands over 128 μg/ml but substancial growth inhibition was observed at concentrations of 4 and 0.06 μg/ml for the *Enterococcus faecalis* reference strain and the vancomycin resistant – ampicillin resistant (VRAR) and vancomycin resistant – ampicillin sensible (VRAS) strains. Moreover, a similar action was observed with *Burkholderia cepacia* SCV at concentrations of 32 μg/ml, but not with the WT strain where the MIC value was higher of 128 μg/ml. These concentrations, lower than MIC and where strong inhibition of the microbial growth was observed, were designated sub-MIC.

The strong bacterial growth inhibition observed at sub-MICs, at concentrations of 7-O-m between 0.06 and 32 μg/ml, encouraged us to further investigate the bacteriostatic activity by studying the kinetics of growth at these concentrations.

Table 15. MICs of the 7-*O*-malonyl macrolactin A and other agents against the target strains.

Target strain	7-O-malonyl macrolactin A		VAN µg/ml	AMP µg/ml	ERY µg/ml	GEN µg/ml	MCZ µg/ml
	MIC µg/ml	Sub-MIC µg/ml					
S. aureus	>128	1	0.125	0.06	0.06	0.25	ND
MRSA2	>128	4	2	0	0	128	ND
MRSA3	>128	4	1	64	0	128	ND
E. faecalis	128	4	2	0.5	2	32	ND
VRAS enterococci	>128	0.06	>128	0.5	128	64	ND
VRAR enterococci	>128	4	>128	>128	2	64	ND
E. coli	>128	-	0	2	32	1	ND
P. aeruginosa	>128	-	0	128	64	128	ND
P. aeruginosa WT	>128	-	0	>128	128	2	ND
P. aeruginosa SCV	>128	-	0	>128	128	8	ND
P. aeruginosa NM	>128	-	0	>128	>128	64	ND
S. maltophilia	>128	-	0	>128	128	16	ND
B. cepacia WT	>128	-	0	>128	>128	>128	ND
B. cepacia SCV	128	32	0	128	128	>128	ND
C. parapsilosis	128	-	0	N/D	N/D	N/D	2
C. krusei	128	32	0	N/D	N/D	N/D	2
C. albicans	>128	-	0	N/D	N/D	N/D	2

Sub-MIC: At these concentrations a strong inhibitory effect in the bacterial growth was observed
ND: not determined
MRSA: methicillin resistant *Staphylococcus aureus*

VRAS: vancomycin resistant-ampicillin sensitive enterococci
VRAR: vancomycin resistant-ampicillin resistant enterococci
WT: wild type
SCV: Small colony variants
CF: Cystic fibrosis
NM: Non mucoid

4.12. Kinetics of growth at sub-MIC of the 7-*O*-malonyl macrolactin A

To further investigate the extent of the bacteriostatic effect of the new macrolactin, growth curves of the target strains were performed at sub-MICs. Specifically, 7-O-m was tested at sub-MIC and 4x sub-MIC values for the microorganisms in which it was detected. The growth curves obtained indicated that the new macrolactin reduced the number of viable cells of *S. aureus* reference strain during the first 4 hours of treatment (Figure 18A). Afterwards, bacterial growth was similar to that of the control but the bacterial concentration in the treated cultures remained 1 $\log_{10}$ lower than the control. Moreover, 7-O-m slightly affected the growth rate of MRSA during the first hours of treatment, and after 4 h of treatment bacterial growth was almost completely inhibited. After 24 h treatment the viable counts of control and treated cultures showed differences of more than 2 $\log_{10}$ (Figure 18B). After 4 h, 7-O-m inhibited the growth of the *E. faecalis* reference strain. Afterwards, the viable counts of the treated bacteria were significant lower than the control culture reaching a maximum difference of 1 $\log_{10}$ at the end of the treatment (Figure 19A). Similarly, for VRAR *E. faecium* strain, no important growth differences were observed during the first hours of 7-O-m treatment but afterwards, macrolactin treatment almost completely inhibited the bacterial growth (Figure 19B).

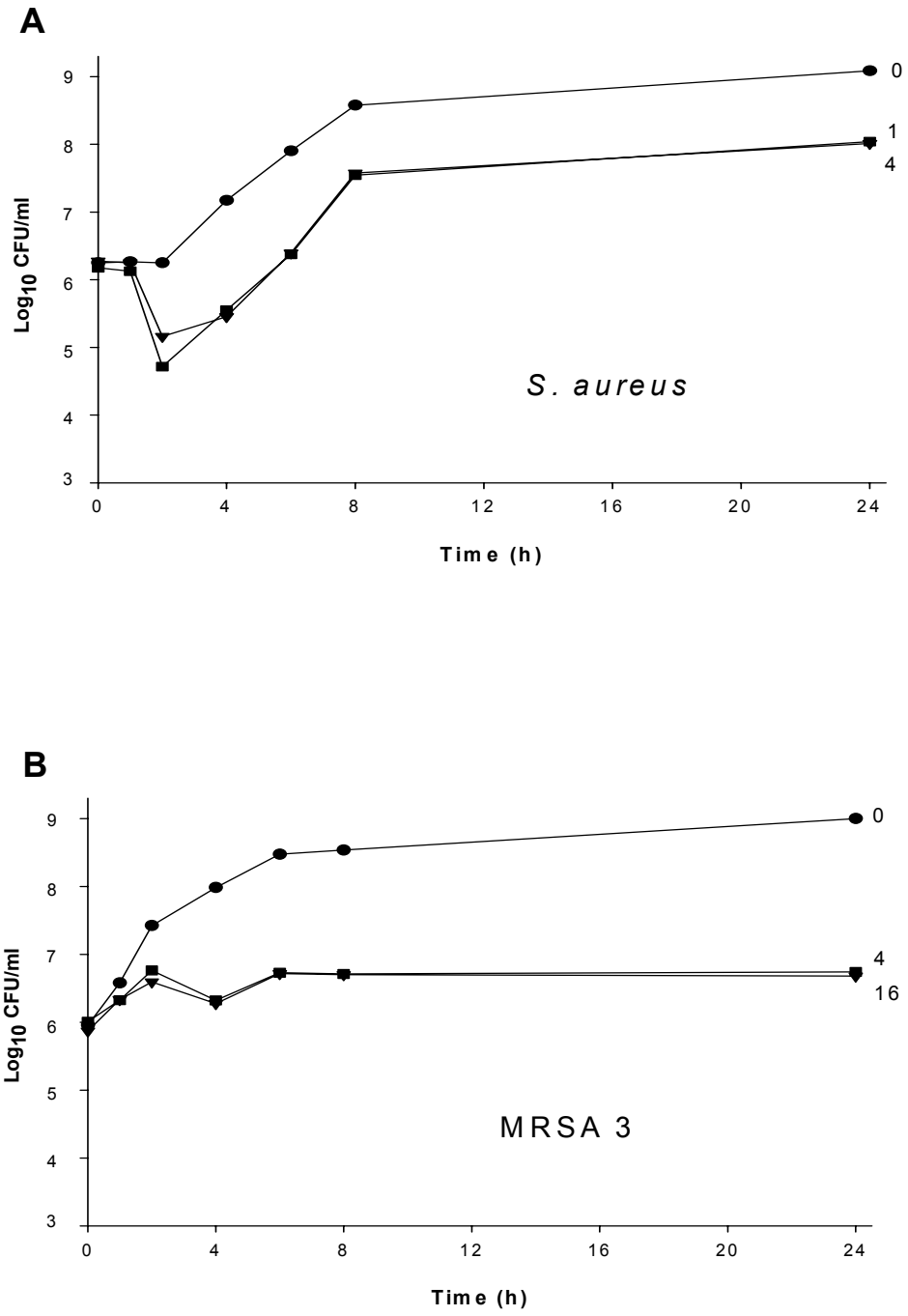
Growth of *Burkholderia cepacia* SCV at sub-MIC was affected directly after starting the macrolactin treatment. In fact, little growth was observed during the first 4-6 hours. The viable counts at the end of the treatment differed 1-1.5 $\log_{10}$ between control and treated

cultures (Figure 20A). Lower activities were observed against *Candida krusei,* the only yeast affected by 7-O-m (Figure 20B), in which the viable counts of the treated bacteria were similar to the control values and only a slight difference was observed between sub-MIC and 4x sub-MIC.

Finally, a concentration-dependent effect was not observed with 7-O-m at 2 different sub-MICs for each strain.

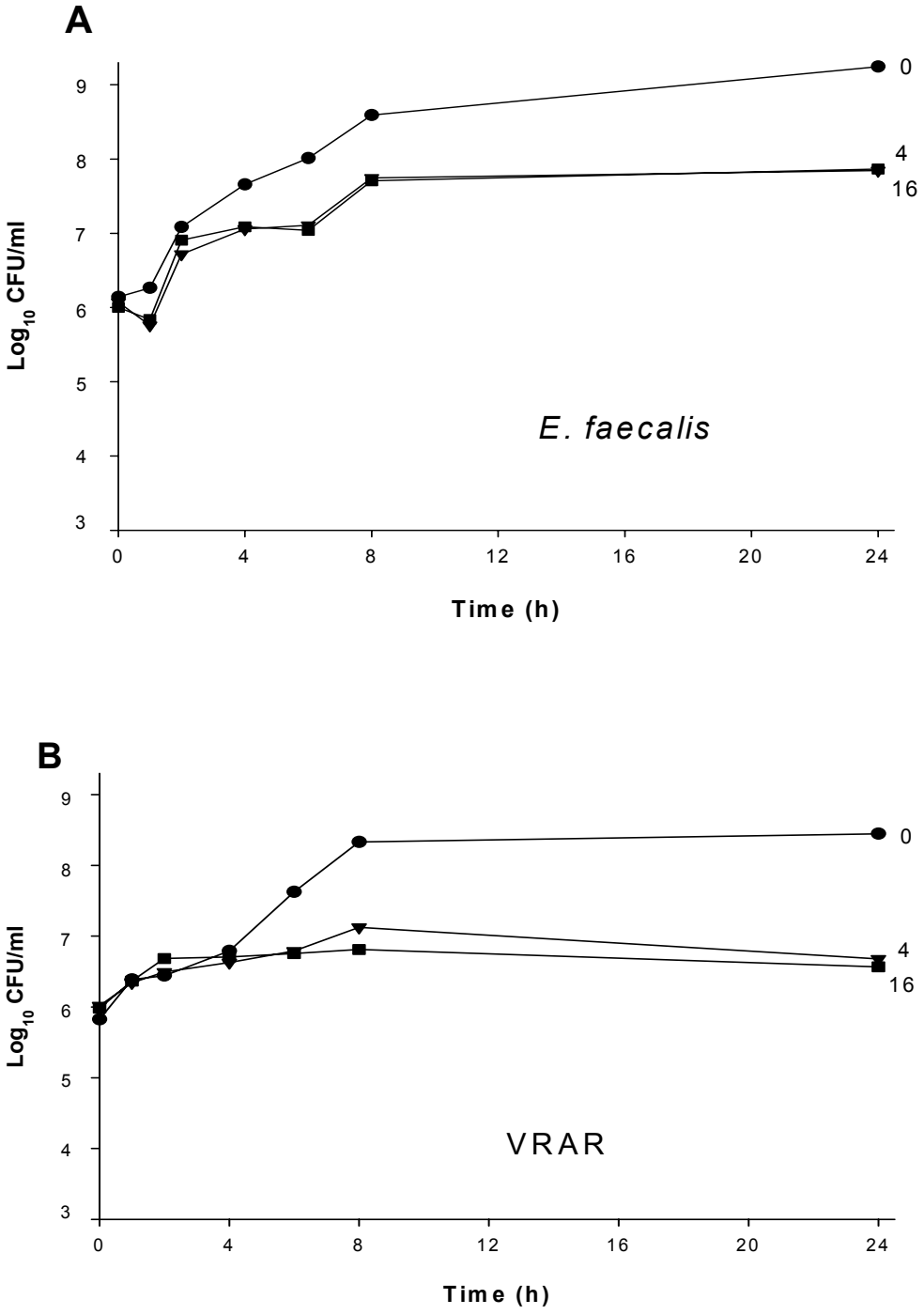
Interestingly, the 7-O-m showed a more pronounced bacteriostatic effect on the growth of resistant gram positive pathogens, where the viable counts do not increase significantly during 24 hours. *B. cepacia* SCV, was the only gram negative strain affected by 7-O-m.

Figure 18. Growth curves of *Staphylococcus aureus* (A) and MRSA 3 (B) in the presence or not of 7-*O*-malonyl macrolactin A



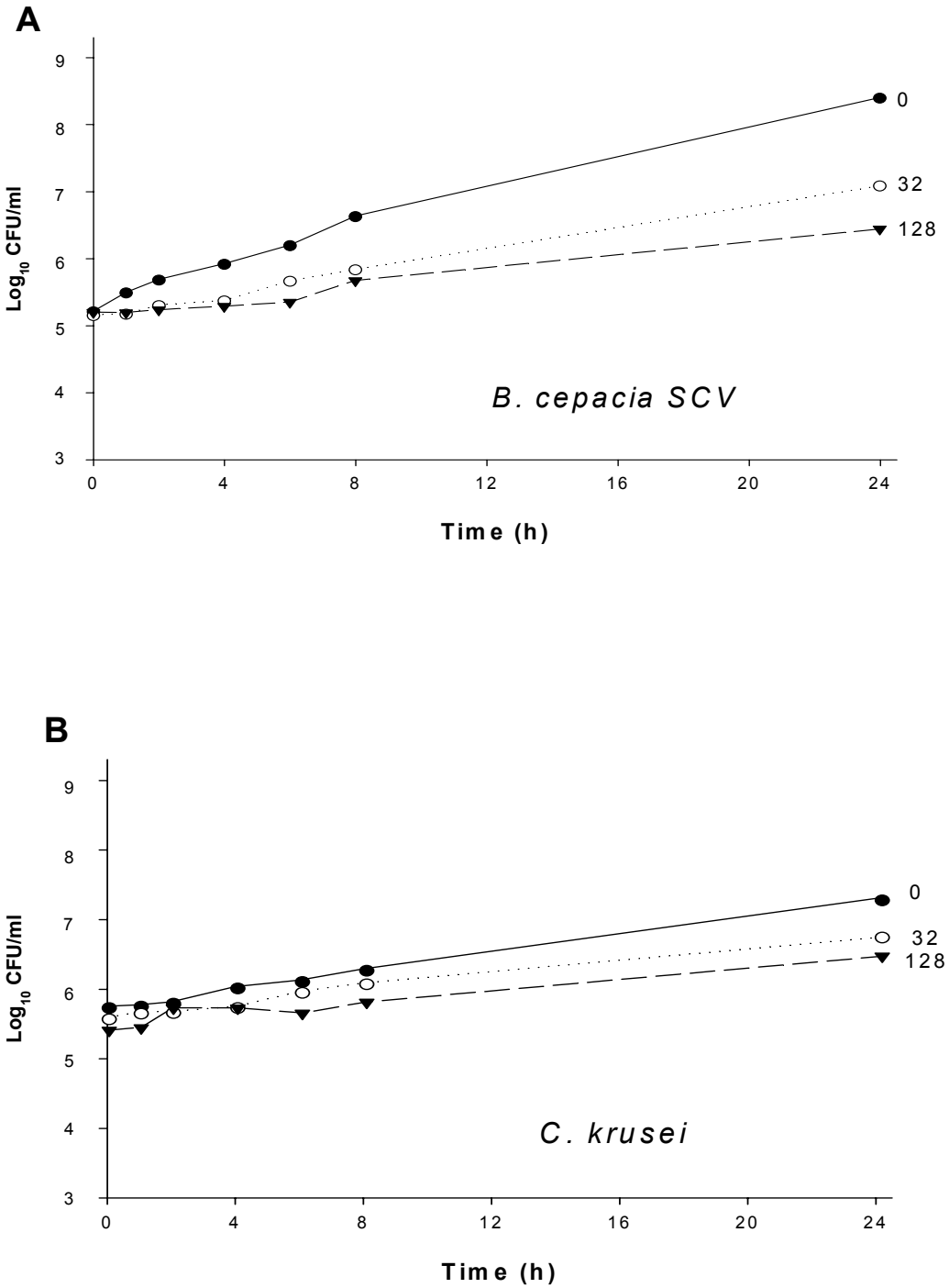
The curve numbers refer to the 7-O-m concentration of the antibiotic in μg/ml and represent control (0), sub-MIC and 4x sub-MIC.
MRSA 3: methicillin resistant *Staphylococcus aureus*

Figure 19. Growth curves of *Enterococcus faecalis* (A) and VRAR (B) in the presence or not of 7-*O*-malonyl macrolactin A



The curve numbers refer to the 7-O-m concentration of the antibiotic in μg/ml and represent control (0), sub-MIC and 4x sub-MIC.
VRAR: vancomycin resistant-ampicillin resistant enterococci

Figure 20. Growth curves of *Burkholderia cepacia* SCV (A) and *Candida krusei* (B) in the presence or not of 7-*O*-malonyl macrolactin A



The curve numbers refer to the 7-O-m concentration of the antibiotic in µg/ml and represent control (0), sub-MIC and 4x sub-MIC.
SCV: Small colony variants

4.13. Post-Antibiotic effect measurements of the 7-O-malonyl macrolactin A

Post-antibiotic effect (PAE) is a term used to describe suppression of bacterial growth that persist after brief exposure of organisms to antimicrobials. The "post-activities" of an antibiotic are not only inhibition of regrowth but also additional effects that include morphological and physiological changes including potential alterations of cellular functions and virulence factors. A prolonged PAE play a beneficial role because during the time that the bacterial pathogen remain inhibited as a result of PAE, it may be sensitive to the lethal effects of patient leukocytes. In addition, the PAE have a clinical impact on antimicrobial dosing regimens. For example, drugs with not PAEs may require more frequent administration than those that demonstrate PAE. The in vitro PAE of 7-O-m with VRAR *E. faecium* and MRSA 3 strain was examined to evaluate the extent of bacterial damage at sub-MIC. The PAE was tested after 1 h of exposure at 16 μg/ml of 7-O-m. The treated MRSA showed a PAE of 0.42 h and the VRAR *E. faecium* a PAE of 2.31 h, indicating that the compound was able to induce a strong damage particularly on enterococci cells even at sub-MICs.

PAE values, were significantly longer with VRAR *E. faecium* than with MRSA 3. During the pre-PAE time, in which the cells were incubated in presence of the antibiotic, the bacteriostatic effect was confirmed, because no growth was detected at this stage.

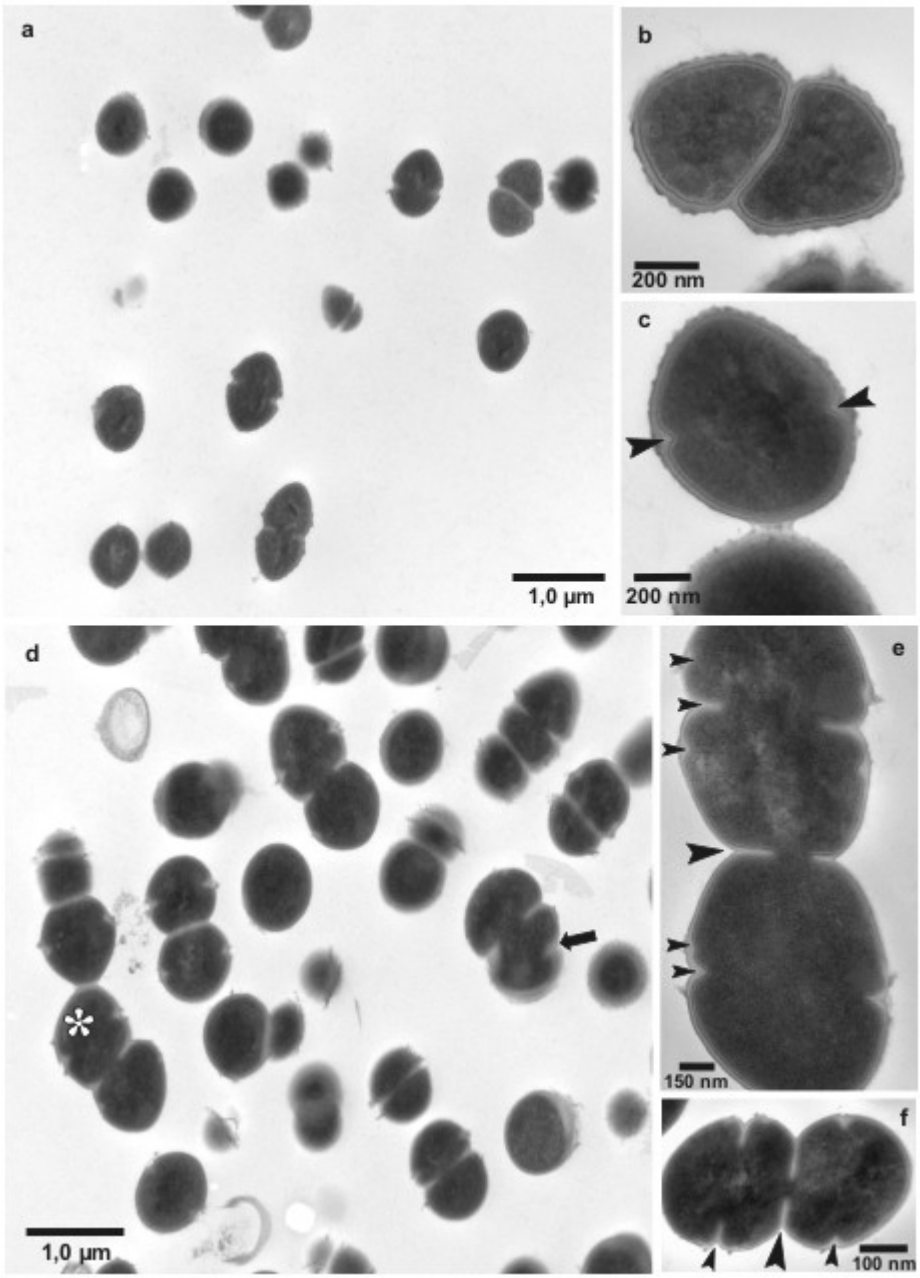
4.14. Morphology alterations in strains treated with sub-MIC of 7-*O*-malonyl macrolactin A

In an attempt to investigated the observed bacteriostatic effect of 7-O-m on the target strains, morphological changes of treated bacteria were monitored by transmission electron

microscopy (TEM). Growth in the presence of 7-O-m had a very marked effect on VRAR *E. faecium* (Figure 21), MRSA3 (Figure 22) and *B. cepacia* SCV (Figure 23) in comparison to untreated controls. Cell division and separation of daughter cells were severely disrupted because of incomplete formation of the septa. In VRAR *E. faecium* (Figure 21) initiation of several symmetric cell divisions can be observed in each cell without finalization of the preceding. Moreover, the rough surface of the control cells was not detected in the treated cells. Similar behaviour was observed with MRSA 3 (Figure 22) but, in this case, the invaginations representing initiation of cell division were not as symmetrical as in VRAR *E. faecium*. Finally, 7-O-m treatment of *Burkholderia cepacia* SCV (Figure 23) resulted in formation of atypical buds in the cell division site and changes in the morphology and dimension of the cells were also observed.

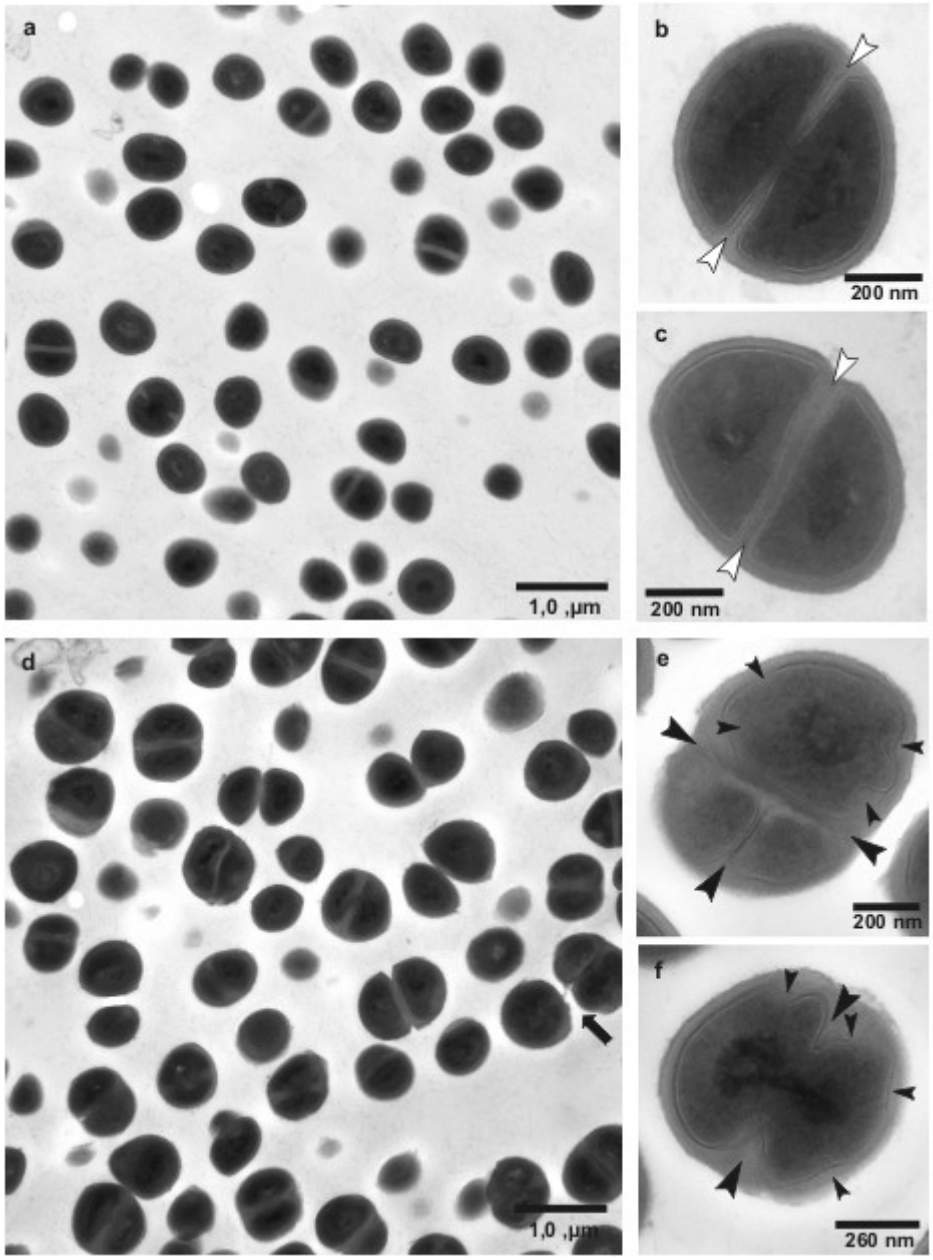
To conclude, TEM observations revealed alterations in the cell division process and morphology of the three pathogens treated with 7-O-m .

Figure 21. Effects of 7-O-malonyl macrolactin A on the morphology of VRAR *E. faecium* clinical isolate.



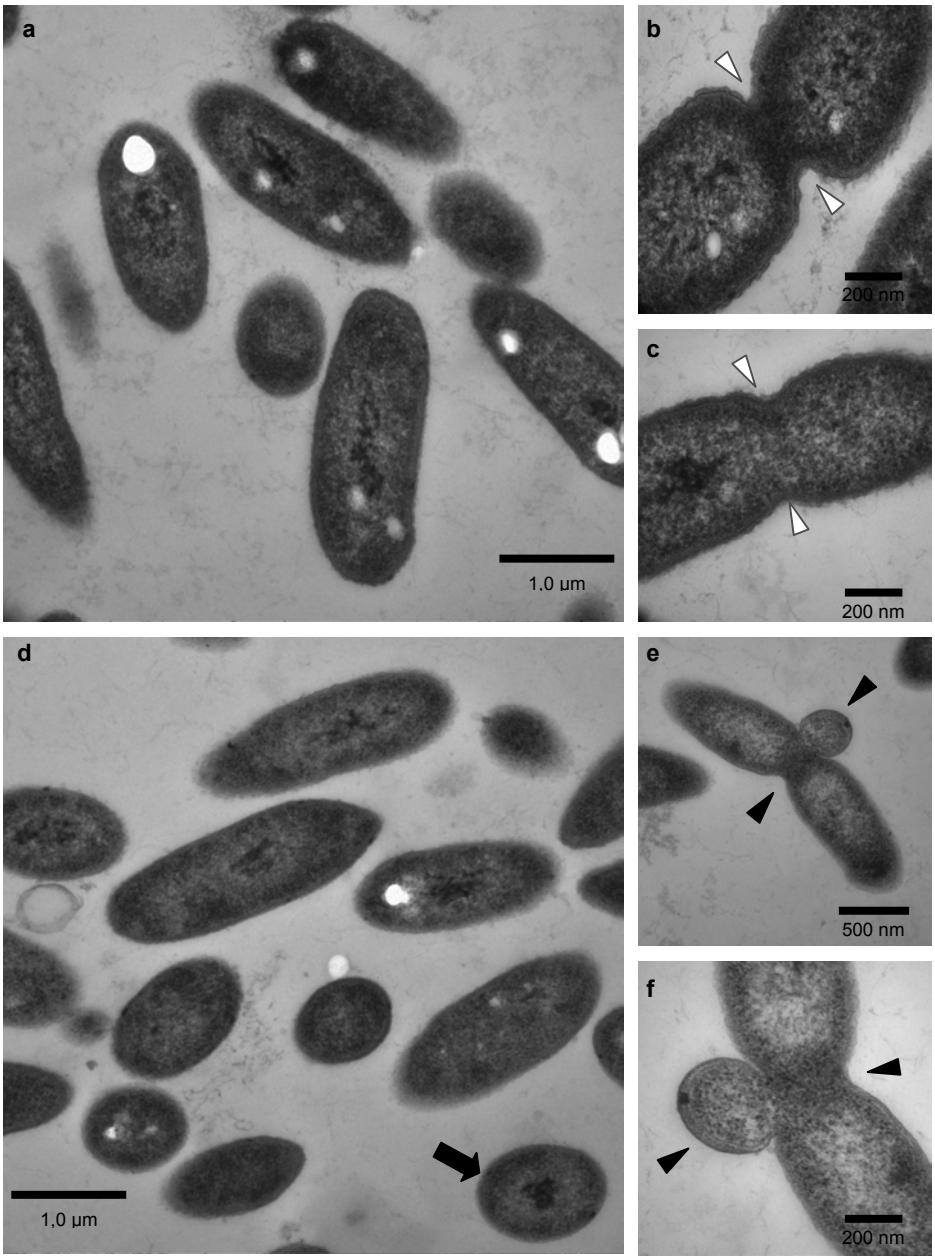
Control (a, b and c) and after 4 h of growth in the presence of 16µg/ml of 7-O-m (d, e and f). The division planes are indicated with large arrowheads. Abnormal division (arrow) and pseudomulticellular chains (white asterisk) with abnormal septa (small arrowheads) without apparent cell division are observed in treated enterococci.

Figure 22. Effects of 7-O-malonyl macrolactin A on the morphology of MRSA 3 clinical isolate.



Control (a, b and c) and after 4 h of growth in the presence of 16µg/ml of 7-O-m (d, e and f). The division plane and cross wall are indicated with white arrowheads in the control cells (b and c). The division planes are indicated with big black arrowheads in the treated bacteria (e and f). Asymmetrical initiation of division is also observed in treated cells (little black arrowheads) in (e and f).

Figure 23. Effects of 7-O-malonyl macrolactin A on the morphology of *Burkholderia cepacia* SCV clinical isolate.



Control (a, b and c) and after 4 h of growth in the presence of 128µg/ml of 7-O-m (d, e and f). The division plane and cross wall are indicated with white arrowheads in the control cells (b and c). The division planes and atypical buds formation are indicated with big black arrowheads in the treated bacteria (e and f). Abnormal size of the cells was observed in treated cells (black arrow in d).

4.15. Eukaryotic cytotoxicity of the 7-*O*-malonyl macrolactin A

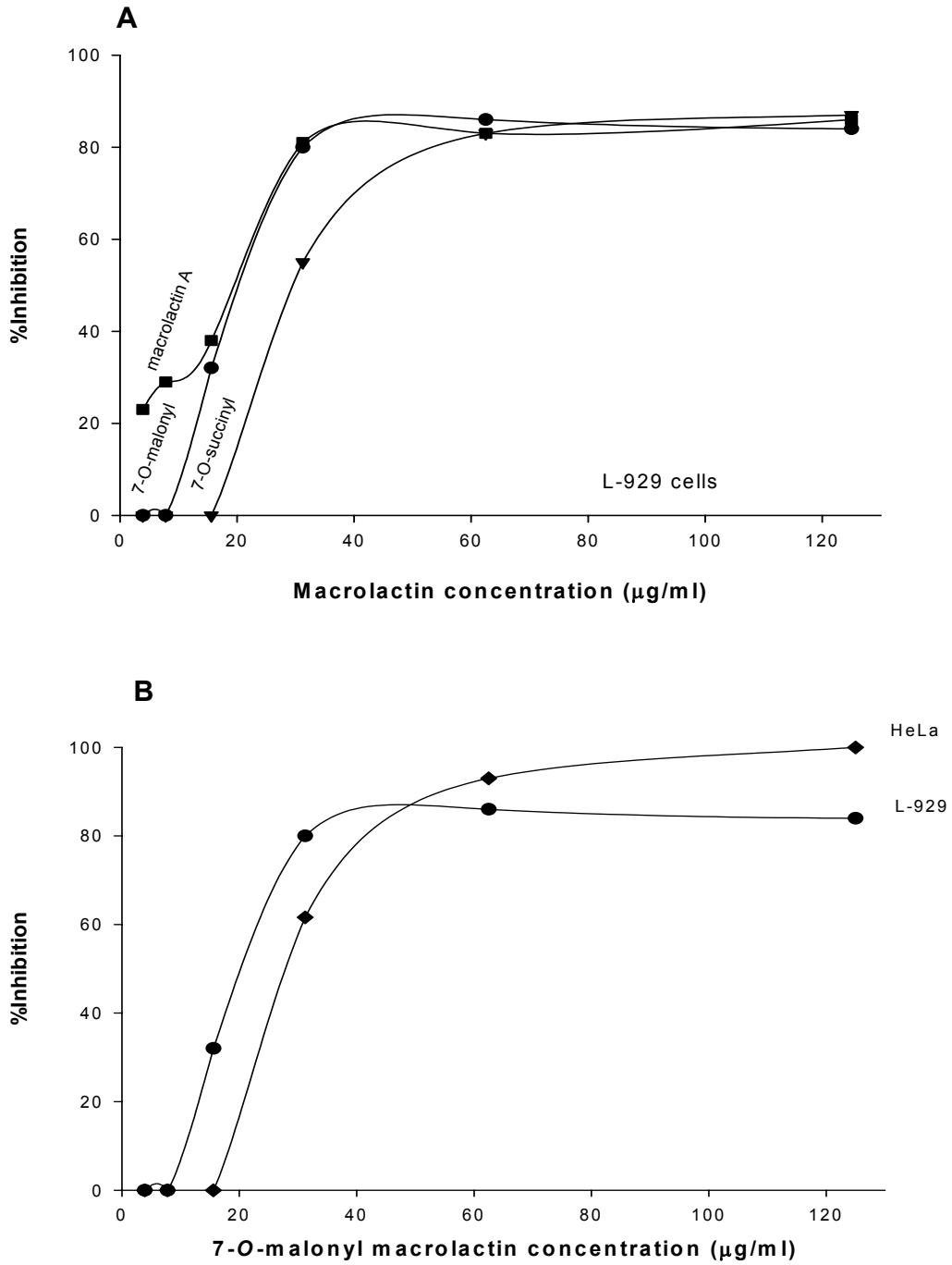
The eukaryotic cytotoxicity assay is used for evaluating the potential toxic properties of the bioactive compounds. Cytotoxicity includes changes in the normal cellular proliferation or morphology. A concern in the development of therapeutic agents is the cytotoxicity of the compound, since it is critically important for antibacterial agents not to be toxic for the host. An agent may have strong antibacterial activity but it would be unacceptable if induce aberrant functions or growth in organ specific tissues.

Cytotoxicity of 7-O-m against mouse fibroblasts and human epithelial cells (L929 and HeLa respectively) was assessed. Microscopical analysis of the macrolactin antiproliferative effect suggested a toxic response because the cellular morphology of the treated cells was round. The control MeOH treated cells where not inhibited. In addition to microscopical analysis, the extent of the cytotoxic effect was quantified using the CyQUANT cell proliferation assay (Molecular Probes), a highly sensitive, fluorescence-based microplate assay for determining numbers of cultured cells [53]. Against L929 cells, the effect of 7-O-m was evaluated in comparison to the other known macrolactins isolated in this study. A small reduction of cell proliferation was observed when the mouse fibroblasts were incubated with 15,6 μg/ml of 7-O-m rising to maximal inhibition (80 %) at 30 μg/ml. Macrolactin A showed higher cytotoxicity compare to the other two compounds (Figure 24A). The human cells were clearly less sensitive to 7-O-m than the mouse cells. The 7-O-m was able to inhibit the proliferation of HeLa cells at a concentration of 31.25 μg/ml. Cells cultures treated with 62.5 μg/ml of 7-O-m showed almost complete inhibition of growth (Figure 24B).

Comparing the action of the new 7-O-m on human epithelial cells and mouse fibroblasts, it was observed that although the compound exert cytotoxicity at 31 μg/ml on both cell lines,

human cell were found to be less sensitive to the drug toxic effect. The 7-O-m and 7-O-s possess lower cytotoxicity, compared with the macrolactin A, a compound known for its antitumoral activities.

Figure 24. Inhibition of L929 cell proliferation by the three macrolactins (A), and comparison of inhibition of eukaryotic cells by 7-*O*-malonyl macrolactin A (B).



4.16. Protein expression of methicillin resistant *Staphylococcus aureus* (MRSA 3) in the presence of 7-*O*-malonyl macrolactin A

Proteomic experiments were carried out to investigate the influence of the 7-O-m in protein expression of the affected pathogens. MRSA 3 proteome changes were analysed following treatment of bacterial cultures with 7-O-m in comparison with untreated cells (Figure 25). The treatment was performed in MH media with 4µg/ml of 7-O-m for 16 hours. This concentration caused a reduction in the growth rate but did not inhibited growth completely. Among 23 identified proteins which showed differences in their expression (Table 16), 15 were expressed only in treated cells (A9–A19 and A21-A24), 7 were found only in untreated cells (C1-C4 and C6-C8) and one protein (C5, A20) was expressed in both, treated and untreated cells, in different amount. The latter protein, afterwards identified as ATP synthetase beta chain, was expressed in less amount in cells treated with 7-O-m.

Sub-MIC of 7-O-m were able to induce important changes in the pattern of expression of several proteins. Of particular interest was the induction of the expression of a cell division protein, since TEM experiments, revealed that 7-O-m induced major changes over the cell division process of pathogenic bacteria.

Figure 25. Two-dimensional gel electrophoresis of protein extracts of methicillin resistant *Staphylococcus aureus* (MRSA 3) treated with and without 7-O-malonyl macrolactin A.

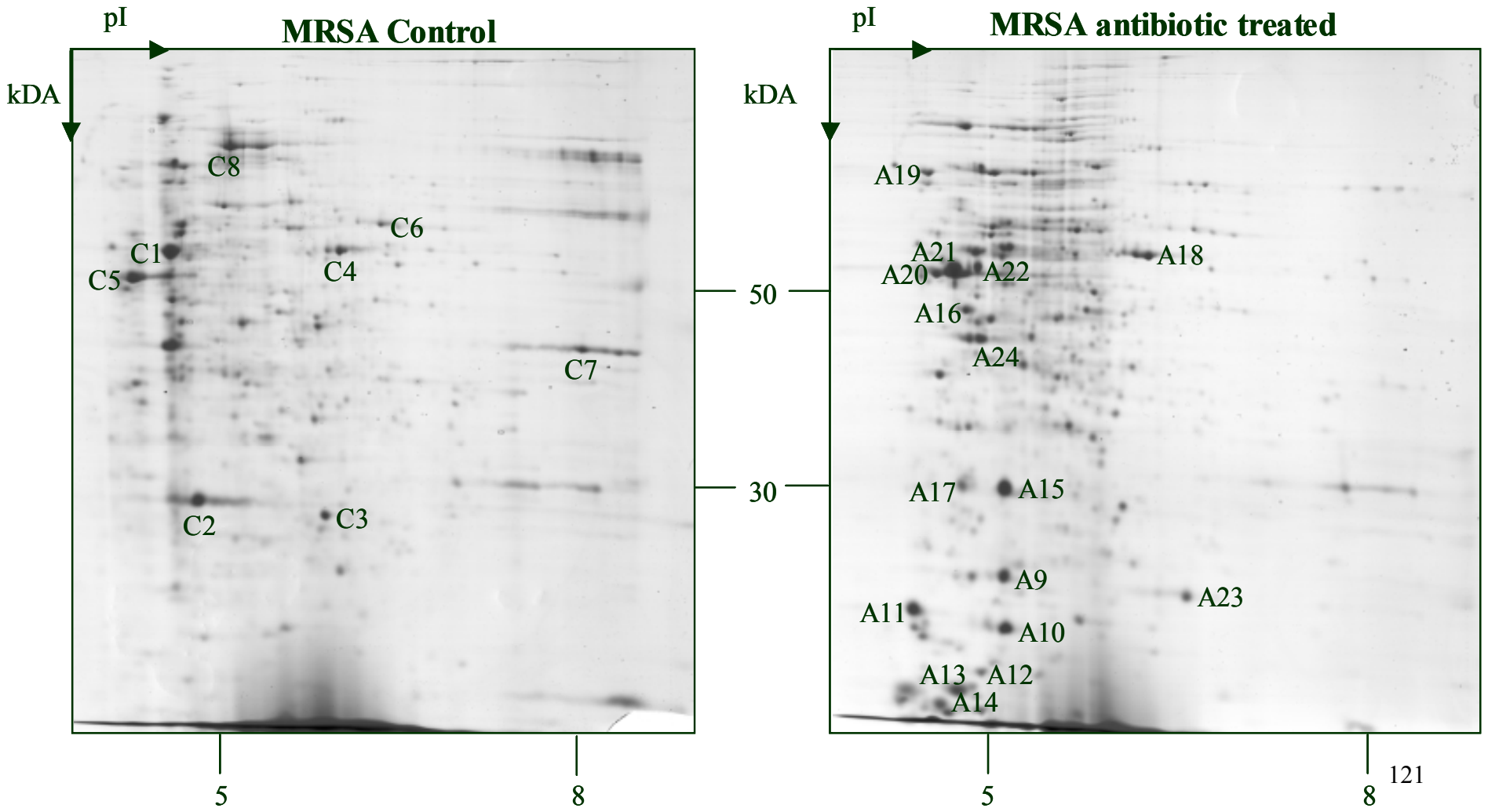


Table 16. Identified Proteins of *Staphylococcus aureus* MRSA 3 treated (A) and not (C) with 7-*O*-malonyl macrolactin A

Spot No.	Proteins	Calculated pI	Theoretical MW (kDa)	Sequence Coverage %
C1	ATP synthase alpha chain	4,91	55,7	26
C2				
C3	transcription pleiotropic repressor	5,87	29,6	44
C4	malate/quinone oxidoreductase	6,12	57,9	27
C5	ATP synthase beta chain	4,68	52,2	51
C6				
C7	Lipase	8,67	45	29
C8				
A9				
A10	alkaline shock protein 23	5,13	19,9	23
A11	glucose-specific enzyme II, PTS system A component resolvase	4,54 9,62	18,4 24,61	42 29
A12				
A13	conserved hypothetical protein	4,76	16,8	67
A14	50S ribosomal protein L7/L12	4,63	13,3	60
A15				
A16				
A17				
A18				
A19				
A20	ATP synthase beta chain	4,68	52,2	55
A21	translational elongation factor TU	4,74	43,9	64
A22	cell division protein	4,87	41,8	36
A23	peptide methionine sulfoxide reductase	6,37	21,4	57
A24	glyceraldehyde-3-phosphate dehydrogenase	4,89	37,6	37

5. DISCUSSION

Secondary metabolites are produced by microorganisms and serve survival functions for the organisms producing them. They act as competitive weapons used against other members of the community: as metal transporting agents; as agents of symbiosis between microbes and plants, nematodes, insects, and higher animals; as sexual hormones; and as regulators of cellular differentiation processes [5, 21, 65, 66]. Furthermore, their production appears to be a response to various ecological pressures within the environment. Microorganisms have shown tremendous potential as producers of novel natural products with important bioactivities, particularly with biomedical roles. Indeed, numerous and useful microbial products have been isolated, including growth inhibitors and promotants, antiviral, antitumor, fungicidal and bactericidal compounds [22, 67]. Despite their broad range of activities, the most studied secondary metabolites through the history have been the antibiotics. Currently, the appearance of multiple antibiotic resistance in pathogenic bacteria is increasing, compromising the clinical treatment of a growing number of infectious diseases. There is thus an urgent need for new drugs effective against current antibiotic resistant pathogens and opportunistic pathogens. However, during the last 20 years no new compounds have been introduced into the clinical practice [18, 68, 69].

The search for active secondary metabolites produced by environmental isolates, using poorly explored microorganisms, could provide a new source for discovering novel bioactive compounds.

This study was mainly aimed at the characterisation of novel secondary metabolites with antibiotic activity against microbial pathogens resistant to antibiotics currently used in the

clinical practice. The vast majority of microorganisms in environmental samples remain unexplored and unknown, since access to this enormous reservoir of secondary metabolites producers has been hindered by the difficulty of culturing most of them. During the first phase of the study, efforts have been devoted to the recovery of secondary metabolites produced from microorganisms isolated from extreme environments. The identification of appropriate culture conditions for optimal production of bioactive metabolites was critical for facilitating the chemical identification, isolation and characterization of the bioactive molecules. Once isolation of the bioactive substance was achieved, structural analysis followed, which revealed whether or not the compounds were new. Subsequent to chemical characterization, determination of bioactivity spectrum of the purified compounds against a selected group of microbial pathogens provided information on the novelty of the activity.

Extracts of cultures of a selected group of 2048 environmental microorganisms, isolated in different geographical habitats, were first screened to detect bioactive substances. Screening tests were performed to detect antibacterial, antiyeast, antifungal activities, as well as effect on eukaryotic cell proliferation and cytotoxicity. Of these extracts, 137 (7 %) obtained from different species belonging to 25 genera, showed interesting and biologically active metabolites, confirming microbial strains as a prolific source for active compounds. Moreover, the great taxonomical variety of the producer microorganisms demonstrates that the production of active secondary metabolites is not restricted to particular genera or species [1, 5, 22, 70]. While strains belonging to unusual genera may provide a higher probability of finding novel bioactive molecules, the fact that they are unusual implies little knowledge about their physiology and appropriate cultivation. Different species of *Arthrobacter, Acinetobacter* and *Rhodococcus*; together with

Micromonospora floridensis, Curtobacter herbarum, Ochrobactrum tritici, in which only one isolate belonging to these species was selected as a producer, had lost the capacity to produce the bioactive compounds when cultivated in a second step. Several strategies of cultivation did not succeed in restoring the production capacity. Isolates of the *Bacillus* genus seem to continue to be promising sources of biologically active secondary metabolites [5], since, the analysis of the bioactive compounds produced by different species allowed to identify a new antibacterial compound. In addition, the experience accumulated with the *Bacillus* strains highlight once more the fact that the production of bioactive secondary metabolites is strain and not species related.

Out of the 137 environmental strains that were selected as producers of active secondary metabolites, 59 % exhibited significant activity against multiresistant and reference strains of gram positive and gram negative bacteria and 58 % were active against fungi and yeasts. Of tested extracts, 8 % showed cytotoxicity against eukaryotic cells in terms of effects on cell morphology and proliferation. The effects induced in eukaryotic cells by the extracts reflects the extent of cytotoxicity of compounds that showed antimicrobial activity, in addition, they indicate potential antitumor activities.

Eighty extracts were selected to assess possible insecticidal activities. Bacteria with toxic activity against insects have been widely investigated as a strategy for biological control of insect pests. *Bacillus thuringiensis* and *Bacillus sphaericus* are most useful bacteria in this regard and have been exploted in insect control for many years. However, the appearance of resistance to its toxins in different mosquitoes species has renewed the interest in the search for alternatives, one of which is new active bacterial products with insecticidal activity [71, 72]. 35 % of the evaluated extracts showed strong larvicidal activity against *Anopheles albimanus (*mosquito vector of human diseases*)* and *Musca domestica*.

Although the number of target strains was low and the concentration of the active compound(s) in the tested extracts was unknown, these results suggest that further investigations are warranted.

As mentioned above, a critical element for the establishment of an efficient production of secondary metabolites is the condition in which the producer microorganism is grown [73]. It have been reported that over 40 % of microorganisms produce active secondary metabolites when they are freshly isolated from nature but this ability is lost in many of them during the storage [5]. Therefore an aim of this study was the establishment of adequate culture conditions to obtain sustained production of the bioactive compounds. 23 % of the strains producing secondary metabolites with antibacterial activity lost this capacity prior to the preparation of a second extract. Several culture conditions and media with different compositions were tested, not generally effective medium for recovery of production of activity was found, even with strains of given species, indicating that the stability of secondary metabolite production is not species related, but, depends on the intrinsic properties of the strain. For example, with a *Bacillus laterosporus* strain after testing several media described in the literature as optimal for the secondary metabolite production in *Bacillus* [23, 63, 74], the capacity to produce compounds with antibacterial activity was recovered in Cooper medium [63]. Different cultivation parameters were used in order to increase the yields of activity in this medium, including changes in all components of the medium, specially Na_2HPO_4, a known critical component for optimal production. Furthermore, several compounds for promoting secondary metabolite production are described in literature like Mg^{+2}, Zn^{+}, Mn^{+2}, Fe^{+}, or substances like DMSO [23, 64, 75, 76]. In this work, the addition of manganese 0.004 % gave the best yield of active compounds produced in Cooper medium by the *B. laterosporus* strain.

Once stable production of secondary metabolites with antimicrobial activity was stablished, and/or the production was enhanced, different approaches to identify and characterise the substances responsible for the activity were investigated. The first methodology implemented was TLC, combined with bioactivity tests, but the little amount of active substances isolated was not sufficient to proceed with the characterisation. On the other hand, TLC was extremely useful to compare the patterns of activities of the different producers strains belonging to the same genera. When selected producers belonged to the same species and showed the same TLC pattern of active compounds, only the extract with the highest activity was further characterised. The second approach was to purify the active compounds using silica gel column chromatography. While this method can be rapid and effective to obtain large quantities of an active compound, it mostly resulted in a sample containing the active substance that needs further purification. HPLC was the third and most successful strategy investigated and became the routine approach for identification of the active compounds contained in bioactive extracts. The correlation between peaks in the chromatogram and biological activity levels in the different HPLC fractions, allowed the identification of the active compounds contained in the extracts. HPLC-UV-MS analysis provided the absorption spectra and masses of the active compounds. Comparison of these data with those of known structures in databases allowed identify compounds as known or unknown, and provided the structures of those that were known.

Over 50 compounds produced by 17 microorganisms with high antibacterial and/or antifungal activity were recognized, mostly known structures. The substances more commonly found were the amicoumacins, antibiotics previously reported with activities against *Staphylocccus aureus* [77]. Other commonly found structures included bacillaene, difficidin and oxydifficidin reported as inhibitors of protein synthesis with activity against

a wide spectrum of bacteria including *Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Serratia marcescens* and *Bacillus thuringiensis* [27, 78]. All the above mentioned antibiotics were presents in the extracts produced by different strains of the *Bacillus* genus. Additionally, bogorols [39], bacillomycins [79-81], actinomycins [82] and moenomycins [83, 84], all known antibacterial compounds produced by *Bacillus* and *Streptomyces* strains were found. Compounds with activity against fungi and yeasts were identified as lagosin and isolagosin and filipins that are produced by *Streptomyces* strains [85, 86]. However, even known compounds can be discovered to posses unknown activities. Recognised known compounds that demonstrate excellent novel in vitro activity with respect to the originally described molecule, particularly against newly isolated resistant pathogens, require additional studies to revaluate their chemical structure and to assess their novelty. The complete characterisation of some active compounds with structures that included polyketides, terpenes and peptides, was not possible because of the low amount produced which was not sufficient for structure elucidation. Particularly with peptides the limitations were the complexity of the characterisation, in some cases their chemical properties exceeded the limit of the available equipment (e.g. molecular masses above 1800) and lack of UV spectra.

A new macrolactin compound with bacteriostatic effect against staphylococci and enterococci, particularly against methicillin resistant *Staphylococcus aureus* and vancomycin resistant enterococci clinical isolates, was discovered and characterised. The producer microorganism was isolated in Indonesia and identified according to pheno and genotypic characteristics as *Bacillus subtilis.* The strain produced three macrolactins and around seven different compounds with antimicrobial activity belonging to the chemical groups of bacillomycins, difficidins and aromatic lipopeptides. Among these, of particular

interest was the new compound 7-*O*-malonyl macrolactin A (7-O-m).

The macrolactins are macrolides containing three separate diene structure elements in a 24-membered lactone ring [62, 87, 88]. These compounds have been reported previously as being products of *Bacillus* sp., unclassifiable marine bacteria and *Actinomadura* sp. [44, 62, 87, 88]. Six macrolactins were first described in 1989, having interesting pharmacological properties with macrolactin A as the most active [87]. In 2000, three new macrolactins with weak antibacterial activities were isolated [62] and in 2001, the isolation of 7 new macrolactins was reported [88]. Until now, sixteen macrolactins have been chemically described and macrolactins A and E have been chemically synthesised [40, 41, 69, 89, 90]. The *Bacillus subtilis* strain analysed during this study produced the known compounds macrolactin A and 7-*O*-succinyl macrolactin A (7-O-s), and the new compound 7-O-m. The structural differences between these three macrolactins are located at residue C-7 and involves a hydroxyl group in macrolactin A, a succinic acid residue in 7-*O*-succinyl and a malonyl group in the new compound 7-O-m.

Macrolactin A has been shown to inhibit murine melanoma cancer cells in *in vitro* assays, to inhibit replication of *Herpes simplex* viruses, to protect T-lymphoblast cells against human HIV viral replication [44, 62, 87, 88], and to inhibit squalene synthase [91].

The macrolactins have been shown to posses antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* [44, 62, 87, 88] and, to our knowledge, no antibacterial activity against multi-resistant clinical isolates has been reported. Nagao *et al*. [88], reported MICs of macrolactins A and F to M between 5 and >100 mg/l, *Staphylococcus aureus* being the most sensitive microorganism tested. Macrolactin A was the most potent antibiotic of the group characterised and showed activity against *Staphylococcus aureus* with MIC of 10 mg/l and 28 mm of inhibition using 100 µg discs [88]. The antimicrobial

activity attributed to macrolactin A varies in the different publications. Gustafson *et al*. [87] reported that macrolactin A inhibited the growth of *Staphylococcus aureus* and *Bacillus subtilis* at concentrations between 5 and 20 µg/disc. Jaruchoktaweechai *et al*. [62] reported that macrolactin A and 7-*O*-Succinyl macrolactin A (7-O-s), exhibited antibacterial activity against the same bacteria with inhibition zones of 8-28 mm at 5-100 µg/disc respectively, but none of the compounds were tested against clinical isolates, particularly multi-resistant strains.

The activities obtained in this study with macrolactin A and 7-*O*-succinyl macrolactin A (7-O-s) produced by *Bacillus subtilis* strain were bacteriostatic in vitro against *Staphylococcus aureus,* with inhibitions zones of approximately 25 mm with 50 µg discs.

The 7-O-m showed higher antibacterial activities particularly against methicillin resistant *Staphylococcus aureus* and vancomycin resistant enterococci strains with inhibition zones of between 15 and 41 mm.

Liquid tests showed that in addition to the MIC, another concentration value (sub-MIC) could clearly be identified, at which a strong inhibition of the bacterial growth was observed. The 7-O-m inhibited strongly the growth of *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus*, *Enterococcus faecalis*, vancomycin resistant enterococci , *Burkholderia cepacia* small colony variants and C*andida krusei*, at concentrations between 0.06 and 32 µg/ml, even though the MICs for all the strains were ≥ 128 µg/ml.

In order to clarify this inhibitory effect, kinetics of growth at these sub-MICs were performed. Interestingly, the 7-O-m showed a more pronounced bacteriostatic effect on the growth of resistant bacteria methicillin resistant *Staphylococcus aureus* and vancomycin resistant enterococci, where the viable counts do not increase significantly over the inoculum values during 24 hours. To our knowledge, there are no studies reporting the

kinetics of growth of strains treated with any of the macrolactins previous characterised.

B. cepacia SCV was the only gram negative strain affected by the 7-O-m, however, the fact that the 7-O-m was able to inhibit the growth of the small colony variants and not the wild type *B. cepacia*, suggests a specific activity against slow growing bacteria, an interesting property that needs further investigation.

The post antibiotic effect (PAE) of 7-*O*-malonyl macrolactin was performed at the sub-MICs values tested in the kinetics studies, in order to further investigate the bacteriostatic activity of the substance at these concentrations. PAE is a term used to describe suppression of bacterial growth that persist after brief exposure of organisms to antimicrobials. A prolonged PAE play a beneficial role because during the time that the bacterial pathogen remain suppressed as a result of PAE, they may be sensitive to the lethal effects of patient leukocytes. PAE values, at sub-MICs, were significantly longer with *E. faecium* vancomycin resistant - ampicillin resistant (VRAR) than with methicillin resistant *Staphylococcus aureus* (MRSA 3). During the pre-PAE time, in which the cells were incubated in presence of the antibiotic, the bacteriostatic effect was confirmed, because no growth was detected at this stage. After removing the antibiotic, the treated *E. faecium* vancomycin resistant - ampicillin resistant (VRAR) was not able to recover the normal growth during a long time (PAE 2.31 h) indicating that the compound, at sub-MICs, induced an extended damage in these bacteria. There are some previous reports in the literature concerning the PAE sub-MIC effects, which seems to be more clinical relevant compared with the PAE, since exposure to supra-inhibitory concentrations will be always followed by sub-MICs in vivo [92]. A long PAE at sub-MICs, considered over 0.30 h, will contribute to the antibacterial effects of an antibiotic. In addition, it is well known, that sub-MICs inhibit bacterial virulence and influence the response of the immune system in

vivo [92].

Transmission electron microscopy observations of the methicillin resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus faecium* strains treated with 7-O-m, revealed alterations in the cell division process, suggesting that separation of the daughter cells was inhibited. Approximately 60 % of the methicillin resistant *Staphylococcus aureus* (MRSA 3) the treated bacteria were observed in packets of not separated cells, lacking complete formation of cells walls, in which multiple asymmetrical initiation points of division were observed. Treatment of *E. faecium* vancomycin resistant - ampicillin resistant (VRAR) with 7-O-m at sub-MICs, induced the formation of chains of not separated bacterial cells in which several septation initiations were observed. Morphology alterations are known to be induced by compounds that inhibit the cell wall synthesis, such as penicillins, and by compound P, the active principle of crude tea extract. Methicillin resistant *Staphylococcus aureus* and *Staphylococcus aureus* cells grown in presence of these compounds, produced a thickened cell wall [93, 94]. Furthermore, abnormal morphology and the appearance of amorphous material on the surface of glycopeptide-treated bacteria were also described for *Staphylococcus aureus* and enterococci [95-97]. Unusually, the formation of buds was observed at the points of cell division in *Burkholderia. cepacia* SCV and cells with abnormal shape and size, smaller and rounded than usual, were also observed. The mechanism by which the 7-O-m exerts its morphological effects, through interfering with cell separation, is interesting because pseudomulticellular clusters are unable to disseminate daughter cells for spreading an infection, and should therefore be less virulent. The characterization of the 7-O-m mode(s) of action, could provide interesting new insights in the search for novel drug targets for future drug discovery.

In addition to the antibacterial activities, macrolactin A has previously been reported to show cytotoxic and antiviral effects [44, 62, 87, 88]. The macrolactin A isolated in this

study showed higher cytotoxicity than the other two macrolactins substituted at C-7 which suggests that the eukaryotic cell toxic effect could be reduced by varying the C-7 substituent.

The 7-O-m studied here, possessed higher antimicrobial activity and lower cytotoxicity than the two known macrolactins produced by the same strain, and other macrolactin variants described in the literature.

The antibacterial activity of 7-O-m was clearly bacteriostatic. Bacteriostatic agents (e.g., β-lactams, chloramphenicol, clindamycin, macrolides and linezolid) have been effectively used for treatment of a range of bacterial infections, including endocarditis, meningitis, and osteomyelitis, indications that are considered to require bactericidal activity [98]. Furthermore, a bacteriostatic agent like clindamycin has been shown to completely inhibit the toxic shock syndrome toxin-1 production by *Staphylococcus aureus* [99] and toxin production in both streptococci and staphylococci [100] suggesting the potential efficacy of bacteriostatic agents in the treatment of infections. It will be interesting to investigate the influence of 7-*O*-m in the production of virulence factors by methicillin resistant *Staphylococcus aureus* and vancomycin resistant enterococci. Since has been clearly demonstrated the efficacy of combination antibiotic therapy, it will be interesting to evaluate synergistic interactions of the 7-O-m with other antibiotics.

Proteomic experiments were carried out to investigate the influence of the 7-O-m on protein expression by methicillin resistant *Staphylococcus aureus*. Different patterns of protein expression were obtained in presence and absence of the 7-O-m. Among 23 identified proteins which showed differences in their expression, 15 were expressed only in treated cells, 7 were found only in untreated cells and one protein was expressed in both, treated and untreated cells, in different amount. Of particular interest was the induction of

expression of a cell division protein, since ultrastructural analysis, revealed that 7-O-m induced major changes in the cell division process of pathogenic bacteria. Further proteomic studies should reveal important insights into the mechanism of action of 7-O-m . During the last years, significant advances have occurred in the field of proteomics that are applicable to antimicrobial lead discovery [101-103]. Furthermore, this method is a recognized tool for the investigation of the action mechanism of antibiotics according to the express for Bandow *et al* [101] and Bruneau *et al* [102].

To conclude, it was described here the discovery and characterisation of a new variant of macrolactin that exhibits important hitherto undescribed clinically relevant activities, namely inhibition of multiresistant gram positive pathogens and of small colony variant of *Burkholderia cepacia*. This compound exerts an interesting effect on cell morphology and appears to inhibit completion of septation during cell division. It shows promise for further development as a new antibiotic and should be further characterised in terms of its mode of action and pharmacological activities.

6. SUMMARY

2048 microorganisms isolated from different environments were evaluated for their production of secondary metabolites with biological activities.

In order to enable preliminary screenings, the strains were cultivated on different culture media according to their origin of isolation. Extracts obtained from 137 strains showed biological activity, of them, 81 were active against microbial targets, 28 exhibited significant activity against insects and 120 were cytotoxic, affecting the morphology or inhibited the proliferation of eukaryotic cells.

The bioactive extracts were chemically analysed and over 50 compounds were recognized as previously reported structures. Three macrolactin compounds were identified, among them, a new structure. These compounds were selected for purification, structure elucidation and investigation of their biological properties.

The new compound, designated 7-*O*-malonyl macrolactin A (7-O-m) and produced by a *Bacillus subtilis* strain isolated from Indonesia, belongs to the macrolactin family, a group of compounds known for their antiviral and cytotoxic properties. When the in vitro antibacterial activities of the three purified macrolactins were tested, the 7-O-m was the most active, particularly against gram positive bacteria. Of great interest, is the capacity of 7-O-m to inhibit the bacterial growth of methicillin resistant *Staphylococcus aureus*, vancomycin resistant enterococci and small colony variants of *Burkholderia cepacia*. Through different biological tests, MIC, sub-MIC, PAE and growth curves in presence of 7-O-m, the nature of the activity was identified as bacteriostatic. The inhibition of the bacterial growth in the presence of 7-O-m was associated with an alteration of the replication process and changes in the pattern of protein expression as observed in TEM

studies and proteomic preliminary studies. 7-O-m shows promise for further development as a new antibiotic and should be further characterised.

7. FUTURE PROSPECTS

Through the history, secondary metabolites have been and continue being an unlimited source of natural products with different activities, one of these, their use as antibiotics. Nowadays, when the antibiotics used in the medical practice have decreased effect over the new isolated multiresistant strains, it is of the special interest to discover new compounds active against resistant pathogens. In this study, 2048 microbial isolates from different environments were evaluated for their production of secondary metabolites with biological activities. In order to further exploit the potential of the extracts produced, other procedures should be developed for the incorporation of new targets into the screening. Furthermore, applications for known antimicrobial compounds could be discover.

The biological activity of the new discovered 7-*O*-malonyl macrolactin A (7-O-m), could be further investigated as follows:

- Evaluation of the activity against other important microbial pathogens as *Mycobacterium tuberculosis* and *Streptococcus* strains.
- Combination of 7-O-m with known antibiotics to evaluated possible synergistic activities against multiresistant pathogens.
- Investigation of the effect in slow growing microorganisms, particularly strains of *Burkholderia cepacia* isolated from cystic fibrosis patients.
- Effect of the 7-O-m on the expression of virulence factors produced by gram positive as well as gram negative bacteria.
- Elucidation of the mechanism of action of 7-O-m using proteomics.
- Identification of the genes involved in the production of 7-O-m .

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9. APPENDIX: MEDIA USED

9.1. OM medium

Starch	1.0 g
Glucose	1.0 g
Peptone	1.0 g
Yeast extract	1.5 g

Distilled water up to 980 ml and autoclave. To solid media add 20 g/l agar. After autoclaved add 10 ml of solutions A and B, and 1 ml of solution C and D.

Solution A

KH_2PO_4	5 g
K_2HPO_4	5 g

Distilled water up to 1000 ml and autoclave.

Solution B

$MgSO_4$	17 g
NaCl	1.0 g
$MnSO_4$	0.7 g
$CuSO_4$	0.06 g

Distilled water up to 1000 ml and autoclave.

Solution C

$FeSO_4.7H_2O$	0.1 g
Na_3-Citrate.$2H_2O$	2.2 g
Ammonium acetate	2 g
Na-thioglycalase	0.75 g
Na_2-succin.$6H_20$	3.3 g

Distilled water up to 100 ml and autoclave by filtering

Solution D	
Biotin	10 mg
Nicotinic acid amide	35 mg
Thiamine HCl	30 mg
p-Aminobenzoid acid	20 mg
Pyridoxal hydrochloride	10 mg
Ca-pantothenate	10 mg
Vitamin B_{12}	5.0 mg

Distilled water up to 100 ml and autoclave by filtering

9.2. Medium X Gen

Glucose	10 g
Peptone	10 g
Yeast extract	15 g
Solution A	10 ml (100 ml: KH_2PO_4 0.50 g, K_2HPO_4 0.50 g)
Solution B	10 ml (100 ml: NaCl 0.10 g, $MgSO_4.7H_2O$ 3 g, $CuSO_4.5H_2O$ 0.01 g, $MnSO_4.5H_2O$ 0.10 g)
Solution C	1 ml (100 ml: $FeSO_4.7H_2O$ 0.10 g, Na_3 citrate 2 g)
solution D	10 ml(100 ml: $CaCl_2.2H_2O$ 0.20 g $ZnCl_2$ 0.20 g).

Distilled water up to 1000 ml and autoclave.

9.3. Bpm (*Bacillus pumilus* medium)

Glucose	20 g
KH_2PO_4	2.0 g
$MgSO_4$.7H2O	0.5 g
NaCl	3.0 g
$FeSO_4$	0.5 mg
Soybean	20 g

Complete the volume to 1L and autoclave.

9.4. Seed Medium

Yeast	12 g
$CaCO_3$	2.0 g
Pharmamedia	10 g
Glycerol	15 g

Distilled water up to 1000 ml and autoclave.

9.5. OMLP (Optimal medium for lipopeptide production)

Sucrose	20 g
KH_2PO_4	1.9 g
$MgSO_4$	0.45 g
KI	0.002 mg
$FeCl_3.6H_2O$	0.005 mg
$MnSO_4.H_2O$	3.6 g
Yeast	7 g
$ZnSO_4.7H_2O$	0.14 mg,
$NaMoO_4$	0.004 mg,
H_3BO_3	0.01 mg,
Peptone	30 g

Distilled water up to 1000 ml and autoclave.

9.6. EM

Yeast extract	5.0 g
Tryptone	20 g
NaCl	5.0 g

Distilled water up to 1000 ml and autoclave. Add glucose 5 g/L.

9.7. Cooper Medium

Glucose	30 g
KH_2PO_4	4.1 g
$MgSO_4$	0.1 g
Na_2HPO_4	5.7 g

$CaCl_2$	0.8 mg
NH_4NO_3	4.0 g

Distilled water up to 1000 ml and autoclave.

9.8. Medium 109 DSM - AOLPA (Artificial Organic Lake peptone agar)

NaCl	100.0 g
$MgCl.6H_2O$	5.0 g
$MgSO_4.7H_2O$	9.5 g
KCl	5.0 g
$CaCl_2.2H_2O$	0.2 g
$(NH4)_2SO_4$	0.1 g
K_2SO_4	0.1 g
Peptone	5.0 g
Yeast Extract	1.0 g

Distilled water up to 960 ml. Adjust the pH to 7.0 + 0.2. Cool to 50°C. Aseptically add 20.0 ml of solution 1 and 20.0 ml of solution 2 and 1.0 ml of solution 3. To solid media add 15 g/l agar.

Solution 1: Hutner's modified salts solution (HMSS)

$C_6H_9NO_6$	10.0 g
$MgSO_4.7H_2O$	29.7 g
$CaCl_2.H_2O$	3.3 g
$Na_2MoO_4.2H_2O$	12.7 mg
$FeSO_4.5H_2O$	99.0 mg
Metals 44 (S1)	50.0 ml

Distilled water up to 1000 ml

Neutralise the $C_6H_9NO_6$ with KOH. Dissolve the remaining ingredients and adjust the pH to 7.0 + 0.2 with KOH or $H2SO_4$. Sterilise at 121°C for 15 minutes and store at 4°C.

Solution 2 : Phosphate supplement (PS)

K_2HPO_4	2.5 g
KH_2PO_4	2.5 g

Distilled water up to 1000 ml

Dissolve and sterilise at 121°C for 15 minutes. Store at 4°C.

Solution 3 : Artificial Organic Lake vitamin solution (AOLV)

Cyanocobalamin	0.1 mg
Biotin	2.0 mg
Calcium Pantothenate	5.0 mg
Folic Acid	2.0 mg
Nicotinamide	5.0 mg
Pyridoxine Hydrochloride	10 mg (Merck 1.07527)
Riboflavin	5.0 mg
Thiamine Hydrochloride	5.0 mg

Distilled water up to 1000 ml. Dissolve and sterilise by filtration (0.22 μm). Store at 4°C.

Metals 44:

Na-EDTA	0.25 g
$ZnSO_4$ .7 H_2O	1.1 g
$FeSO_4$ .7 H_2O	0.5 g
$MnSO_4$ .H_2O	0.15 g
$CuSO_4$ .5 H_2O	0.04 g
$Co(NO_3)_2$. H_2O	0.024 g
$Na_2B_4O_7$ .10 H_2O	0.017 g
Distilled water	100 ml

Dissolve the EDTA and add a few drops of concentrated H_2SO_4 to retard precipitation of the heavy metal ions.

9.9. Enrichment Medium

Na_2CO_3	3.0 g
KCl	2.0 g
$MgSO_4.7H_2O$	1.0 g
NaCl	100 g
Yeast Extract	10 g

$MnCl_2.4H_2O$	0.36 mg
$FeSO_4$	50 mg

Distilled water up to 1000 ml and autoclave.

9.10. Mueller Hinton Agar (MH)

MH agar	38.0 g

Distilled water up to 1000 ml and autoclave.

9.11. Mueller Hinton Broth (MH)

MH Broth	21.0 g

Distilled water up to 1000 ml and autoclave.

9.12. Medium 61 DSM (M61)

Tryptone	10 g
Sucrose	10 g
Yeast extract	2 g
$FeSO_4.7H_2O$	0.2 g
Na_2SO_3	0.2 g
$Na_2S_2O_3.5H_2O$	0.08 g

Distilled water up to 1000 ml and autoclave. pH: 6.8 –7.8

Add after autoclaved 1 ml/10 ml medium of each one of this substances:

$NaHCO_3$	4.2 g/100 ml
Na_2CO_3	5.3 g/100 ml

9.13. Medium 284 DSM (*Halomonas pantelleriensis*)

Yeast extract	10 g
Na citrate	3.0 g
KCl	100 g
NaCl	100 g
Na_2CO_3	100 g
$MgSO_4.7H_2O$	1.0 g

Distilled water up to 1000 ml and autoclave.

9.14. GEN medium

Glucose	2.0 g
Yeast extract	3.0 g
NaCl	1.0 g

Distilled water up to 1000 ml and autoclave.

9.15. SMM (Synthetic Malate medium)

DL-Malic acid	5.0 g
KOH	4.5 g
KH_2PO_4	0.6 g
K_2HPO_4	0.4 g
$MgSO_4.7H_2O$	0.2 g
NaCl	2.5 g
$CaCl_2$	0.02 g
$MnSO_4.H_2O$	2.0 mg
FeEDTA (0.66 w/v)	10 ml
Biotin	0.1 mg
NH4Cl	0.5 g
Yeast extract	0.1 g

Distilled water up to 1000 ml. Adjust pH to 7.2.

9.16. ANA (Alkaline Nutrient Agar)

Peptone	5.0 g
Meat extract	3.0 g

Distilled water up to 1000 ml. After sterilization add sterile 1 M Na-sesquicarbonate solution (1 ml in 10 ml) to achieve a pH of 9.7.

Na-sesquicarbonate solution:

$NaHCO_3$	4.2 g
Na_2CO_3 anhydrous	5.3 g

Distilled water up to 1000 ml.

9.17. Landy Medium

Glucose	20 g
KH_2PO_4	1.0 g
$MgSO_4.7H_20$	0.5 g
$FeSO_4.6H_2O$	$1.2x10^{-3}$ g
$CuSO_4.5H_2O$	$1.6x10^{-3}$ g
$MnSO_4$	$0.4x10^{-3}$ g
L-Glutamic acid	5.0 g
Yeast extract	10 g

Distilled water up to 1000 ml. Adjust pH: 7.5

9.18. Difco Nutrient broth (DSM)

$MgSO_4$	10 ml of a solution 1.2 %
KCl	10 ml of a solution 10 %
$FeSO_4$	0.15 g
$MnCl_2$	1.3 g
NaOH	40 g
$Ca(NO_3)_2$	16.4 g

9.19. NBG

Nutrient Broth	8.0 g
Glucose	5.0 g

Distilled water up to 1000 ml and autoclave.

9.20. Medium 90 DSM - Malt Extract Peptone Agar

Malt extract	30 g
Soya peptone	3.0 g

Distilled water up to 1000 ml. Adjust pH to 5.6. Sterilize at 121°C for 10 min. To solid media add 15 g/l agar.

9.21. Medium 220 DSM - CASO AGAR

Peptone from casein	15 g
Peptone from soy meal	5.0 g
NaCl	5.0 g

Adjust pH to 7.3. Distilled water up to 1000 ml and autoclave. To solid media add 15 g/l agar.

9.22. Medium 545 DSM - TSB (Tryptone soy broth)

Peptone from casein	17.0 g
Peptone from soy meal	3.0 g
D(+)-Glucose	2.5 g
NaCl	5.0 g
K_2HPO_4	2.5 g

Adjust pH to 7.3.Distilled water up to 1000 ml and autoclave.

9.23. MSAM (Minimal Salt Agar Medium)

$MgSO_4.7H_2O$	0.5 g
K_2HPO_4	0.7 g
KH_2PO_4	0.3 g
$FeSO_4.H_2O$	0.01 g
$ZnSO_4$	0.001 g
$MnCl_2$	0.001 g

Distilled water up to 1000 ml and autoclave.

9.24. BHI (Brain heart infusion)

Brain Heart Infusion	18.5 g

Distilled water up to 1000 ml and autoclave.

9.25. St-1 (*Streptomyces* medium 1)

Maltose	10 g
Glycine	4.5 g
K_2HPO_4	0.12 g

KH_2PO_4	0.12 g
NaCl	1.0 g
$CaCl_2$	0.1 g
Traces	20 ml

Distilled water up to 1000 ml and autoclave.

Traces:

$FeSO_4.7H_2O$	1.0 g
$MnCl_2.4H_2O$	1.0 g
$ZnSO_4.H_2O$	1.0 g
$CaCl_2$	1.0 g

Distilled water up to 100 ml.

9.26. St-2 (*Streptomyces* medium 2)

$MgSO_4.7H_2O$	0.6 g
K_2HPO_4	3.5 g
L-Asparagine	2.0 g
Glycerol	10 ml
MOPS buffer	21 ml
Traces	1 ml

Distilled water up to 1000 ml and autoclave. Adjust pH 6.8.

9.27. St-3 (*Streptomyces* medium 3)

Glucose	75 g
NH_4Cl	9.0 g
K_2HPO_4	1.0 g
MSG	5.0 g
$CaCO_3$	5.0 g
MOPS	21
Salt 1	20 ml

Distilled water up to 1000 ml and autoclave.

Salt 1:

$MgSO_4.7H_2O$	28,9 g
$FeSO_4.7H_2O$	0.5 g
$ZnSO_4.7H_2O$	0.5 g
$MnSO_4.H_2O$	0.1 g
$CuSO_4.5H_2O$	0.05 g
$CaCl_2.6H_2O$	0.04 g

Distilled water up to 1000 ml.

9.28. St-4 (*Streptomyces* medium 4)

Tryptone	17 g
Soy peptone	3.0 g
NaCl	5.0 g
K_2HPO_4	1.25 g

Distilled water up to 1000 ml and autoclave.

9.29. St-6 (*Streptomyces* medium 6)

Soy bean meal	20 g
Casamino acids	10 g
Yeast extract	1.0 g
Meat extract	1.0 g
Lysine HCl	1.0 g
$Na_2S_2O_3.5H_2O$	0.5 g
$MgSO_4.7H_2O$	0.5 g
K_2HPO_4	0.5 g

Distilled water up to 1000 ml and autoclave.

9.30. Mic-1 (*Micromonospora* natural medium 1)

Beef extract	3.0 g
Tryptone	5.0 g
Glucose	8.0 g
Starch	24 g

Yeast extract	8.0 g
$CaCO_3$	1.0 g
$NaNO_3$	2.0 g
$Ca(NO_3)_2$	1.0 g
$Co(NO_3)_2.6H_2O$	5x10-3 g
$(NH_4)_6(Mo_7O_{24}).4H_2O$	2x10-4 g
$FeSO_4.7H_2O$	5x10-3 g
$CuCl_2.2H_2O$	2x10-4 g
$ZnCl_2$	2x10-4 g
$MnSO_4.4H_2O$	3x10-3 g

Distilled water up to 1000 ml and autoclave.

9.31. Mic-10 (*Micromonospora* medium 10)

Glucose	10 g
Starch	24 g
Peptone	3.0 g
Meat extract	3.0 g
Yeast extract	5.0 g
$CaCO_3$	4.0 g

Distilled water up to 1000 ml and autoclave.

9.32. Mic-15 (*Micromonospora* medium 15)

Soluble starch	20 g
Glucose	5.0 g
NH_4Cl	5.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
$CaCO_3$	1.0 g
NH_4NO_3	4.0 g
$(NH_4)_2SO_4$	6.5 g

Distilled water up to 1000 ml and autoclave.

9.33. PM-5

Dextrin	12 g
Cottonseed flour	30 g
Wheat germ	12 g
Dry yeast	1.2 g
$CaCO_3$	6.0 g
$MgSO_4$	6.0 g
K_2HPO_4	2.4 g
$FeSO_4.7H_2O$	0.8 g
$CoCl_2.6H_2O$	0.02 g

Distilled water up to 1000 ml and adjust pH 7.2 with 1N NaOH.

9.34. Medium 125: *Methylobacterium* medium

KNO_3	1.0 g
$MgSO_4.7\ H_2O$	0.2 g
$CaCl_2\ .\ 2\ H_2O$	0.02 g
Na_2HPO_4	0.23 g
NaH_2PO_4	0.07 g
$FeSO_4\ .7\ H_2O$	1.0 mg
$CuSO_4\ .5\ H_2O$	5.0µg
H_3BO_3	10µg
$MnSO_4\ .5\ H_2O$	10µg
$ZnSO_4\ .7H_2O$	70µg
MoO_3	10µg

Distilled water up to 1000 ml. Add 5 ml MeOH or incubate under methane-air (4:1). Adjust pH to 6.8. To solid media add 12 g/l agar.

9.35. Medium 457 DSM - Mineral medium (Brunner)

Na_2HPO_4	2.4 g
KH_2PO_4	1.5 g
$(NH_4)_2SO_4$	0.5 g

$MgSO_4 . 7 H_2O$	0.2 g
$CaCl_2 . 2 H_2O$	0.05 g
Trace element sol. SL-4	10 ml

Distilled water up to 1000 ml. Adjust pH to 6.9.

Trace element solution SL-4:

EDTA	0.50 g
$FeSO_4 . 7 H_2O$	0.20 g
Trace element solution SL-6	100 ml

Distilled water up to 1000 ml

9.36. Trace element solution SL-6:

$ZnSO_4 . 7 H_2O$	0.10 g
$MnCl_2 . 4 H_2O$	0.03 g
H_3BO_3	0.30 g
$CoCl_2 . 6 H_2O$	0.20 g
$CuCl_2 . 2 H_2O$	0.01 g
$NiCl_2 . 6 H_2O$	0.02 g
$Na_2MoO_4 . 2 H_2O$	0.03 g

Distilled water up to 1000 ml.

9.37. Medium 606 DSM - Colby and Zathman medium

K_2HPO_4	1.20 g
KH_2PO_4	0.62 g
$CaCl_2 . 6 H_2O$	0.05 g
$MgSO_4 . 7 H_2O$	0.20 g
NaCl	0.10 g
$FeCl_3 . 6 H_2O$	1.0 mg
$(NH_4)_2SO_4$	0.5 g
$CuSO_4 . 5 H_2O$	5.0µg
$MnSO_4 . 5 H_2O$	10µg
$Na_2MoO_4 . 2 H_2O$	10µg

H_3BO_3	10µg
$ZnSO_4$. 7 H_2O	70µg
$CoCl_2$. 6 H_2O	5.0µg
Purified agar	15 g
MeOH	2.0 ml

Distilled water up to 1000 ml. Adjust pH to 7.0. Autoclave at 121°C for 15 min. Cool to 50°C. Add a filter-sterilized solution of MeOH to give a final concentration of 0.2 %.

9.38. Medium 805 DSM - *Methylobacterium thiocyanatum* medium

KSCN	0.25 g
$MgSO_4$. 7 H_2O	0.10 g
K_2HPO_4	1.50 g
Na_2HPO_4 . 2 H_2O	7.90 g
Glucose	4.50 g
$FeSO_4$. 7 H_2O (2 %)	1.0 ml

Distilled water up to 1000 ml. To solid media add 15 g/l agar. Sterilize medium at 115°C for 10 minutes.

9.39. Alc-1 (*Alcaligenes* medium 1)

Na_2HPO_4	9.0 g
KH_2PO_4	1.5 g
NH_4Cl	1.0 g
$MgSO_4.7H_2O$	0.2 g
Fe NH_4 Citrate	1.2 mg
$CaCl_2$	20 mg
Hoagland Solution	2.0 ml
$NaHCO_3$	0.5 g

Distilled water up to 1000 ml and autoclave.

9.40. Alc-2 (*Alcaligenes* medium 2)

Glucose Monohydrate	10 g
Yeast extract	1.0 g

Polypeptone	10 g
Globe corn starch	20 g

Distilled water up to 1000 ml and autoclave.

9.41. KMA (King medium A)

Peptone	20 g
Glycerol	10 ml
K_2SO_4	10 g
$MgCl_2$	10 g

Distilled water up to 1000 ml and autoclave.

9.42. KMB (King medium B)

Protease peptone n.3	20 g
Glycerol	10 ml
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g

Distilled water up to 1000 ml and autoclave.

9.43. YSE

Ethanol	15 g
Yeast extract	3.0 g
Soy flour	3.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
NaCl	2.0 g
$CaCO_3$	2.0 g

Distilled water up to 1000 ml and autoclave.

9.44. F1 medium

Bactopeptone	10 g
Yeast extract	10 g
Glucose	30 g

Distilled water up to 1000 ml and autoclave.

9.45. F2 medium

Glycerol	10 g
Sodium glutamate	10 g
Yeast extract	30 g

Distilled water up to 1000 ml and autoclave.

9.46. Luria Broth (LB)

Yeast extract	5.0 g
Tryptone	20 g
NaCl	5 g

Distilled water up to 1000 ml and autoclave. To solid media add 15 g/l agar.

9.47. R2A

Yeast extract	0.5 g
Peptone	0.5 g
Casamino acids	0.5 g
Glucose	0.5 g
Starch	0.5 g
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.05 g
Sodium pyruvate	0.3 g

Distilled water up to 1000 ml and autoclave.

9.48. BLPM (*Bacillus laterosporus* production medium)

Glycerol	15 g
Pharmamedia	10 g
Dry yeast	12 g
L-Leucine	5.0 g
L-Histidine-HCl	2.0 g
$CaCO_3$	2.0 g

Distilled water up to 1000 ml and autoclave. pH:7.0

9.49. Vegetative medium

Glycerol	20 g
Pharmamedia	10 g
Corn Steep liquor	10 g
(NH4)2SO4	3.0 g
CaCO3	4.0 g
ZnSO4.7H2O	0.3 g

9.50. TSYEM

Trypticase soy broth	30 g
Yeast extract	3 g

Distilled water up to 1000 ml and autoclave.

9.51. Medium 65 DSM - Gym *Streptomyces* medium

Glucose	4.0 g
Yeast extract	4.0 g
Malt extract	10 g
$CaCO_3$	2.0 g

Distilled water up to 1000 ml and autoclave. To solid media add 12 g/l agar.
Adjust pH to 7.2 with KOH before adding agar (use pH-indicator paper). Delete CaCO3 if liquid medium is used.

9.52. YEG

Yeast Extract	5.0 g
Glucose	1.0 g

Distilled water up to 1000 ml and autoclave.